



The role of proteolysis in the modulation of nitrogen metabolite repression in *Aspergillus nidulans*

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DECLARATION

I certify that this thesis contains my own work, except where acknowledged, and that no part of this material has been previously submitted for a degree or any qualification at this or any other university.

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ABBREVIATIONS

<i>A. nidulans</i>	<i>Aspergillus nidulans</i>
aa	Amino acids
AMM	<i>Aspergillus nidulans</i> minimal media
ARF	Auxin response factor
AmSO ₄	Ammonium sulphate
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CCR	Carbon catabolite repression
CD	Circular dichroism
DBD	DNA binding domain
dH ₂ O	Deionised water
DHQase	dehydroquinase
DNA	Deoxyribonucleic acid
ds	Double strand
DSC	Differential scanning calorimetry
DTT	DL-dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diaminetetra-acetic acid
EST	Expressed sequence tag
FPLC	Fast protein liquid chromatography
HRP	Horseradish peroxidase
FP	Fluorescence polarisation
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl β-D-thiogalactoside
ITC	Isothermal titration calorimetry
MOPS	Morpholinopropanesulphonic acid
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADP-GDH	NADP-glutamate dehydrogenase
NCR	Nitrogen catabolite repression (<i>S. cerevisiae</i>)
NMR	Nitrogen metabolite repression (<i>A. nidulans</i> and <i>N. crassa</i>)
<i>N. crassa</i>	<i>Neurospora crassa</i>
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPD	Proteolytic processing domain
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDR	Short chain dehydrogenase/ reductase
SDS	Sodium dodecyl sulphate
TEMED	N, N, N', N' – tetramethyl ethylenediamine
UV	Ultra violet
UAR	Upstream activation region
UTR	Untranslated region
Zf	Zinc finger

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ABSTRACT

When *Aspergillus nidulans* grows with ammonium or glutamine combined with an alternative nitrogen source, the organism preferentially utilises the ammonium or glutamine. The pathways for utilising alternative nitrogen source remains inactive in the presence of ammonium or glutamine and this phenomenon is called nitrogen metabolite repression (NMR). The regulation of NMR in *A. nidulans* is mediated by multiple mechanisms which include transcription, mRNA stability, translation, posttranslational modification and direct protein-protein interactions. A key feature of NMR in *A. nidulans* is the interaction between the GATA type transcription activator AreA and the transcription repressor protein NmrA.

The transcription repressor NmrA of *A. nidulans* discriminates between oxidised and reduced dinucleotides; however dinucleotide binding has no effect on its interaction with the zinc finger in the transcription activator AreA. The role of specific cleavage of transcription repressor proteins by proteases and how this may be related to the emerging theme of dinucleotides as cellular signalling molecules is poorly characterised. Protease activity in *A. nidulans* was assayed using NmrA as the substrate, and was absent in mycelia grown under nitrogen sufficient conditions but abundant in mycelia starved of nitrogen. Three proteases were identified and two were purified and identified by mass spectroscopy as serine proteases: Q5BGU2_EMENI and Q5BAR4_EMENI, encoded by the genes AN0238.2 and AN2366.2, respectively. Production of the third protease was absent in strain deleted for the *areA* gene.

Proteolysis of NmrA occurred in an ordered manner by preferential digestion within a C-terminal surface exposed loop and subsequent digestion at other sites. The two proteolytic fragments of NmrA produced by digestion at the C-terminal site remained associated; however the digested NmrA was unable to bind to the AreA zinc finger but retained the ability to bind NAD^+ . These data reveal a potential new layer of control of nitrogen metabolite repression by the ordered proteolytic cleavage of NmrA. NmrA digested at the C-terminal site showed a resistance to further digestion that was enhanced by the presence of NAD^+ and to a lesser extent by NADP^+ . This is the first time that an effect of dinucleotide binding to NmrA has been demonstrated.

The *in vitro* ordered proteolysis of NmrA reveals a potential new level of regulation relating to nitrogen metabolite repression. The dynamic interplay between the production of NmrA and its subsequent ordered proteolysis facilitates a rapid and finely tuned response to changes in the concentration and nature of the nitrogen source supporting growth.

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Chapter 1 Introduction

1.1 Fungi

Fungi are commonly defined as a eukaryotic group separated from plants, animals and bacteria. Fungi (yeast, mushrooms etc) reproduce through the production of sexual and asexual spores and lives by absorbing nutrients from surrounding environment like plants and bacteria. As their shared features, such as growth immobility, cell wall formation and sexual/ asexual lifestyle, fungi had been classified as plants for many years until the five kingdom classification (Monera, Protista, Fungi, Animalia, Plantae) was proposed by Whittaker (Whittaker and Margulis, 1978). The biggest difference of fungi to plant is the lack of chlorophyll and thus the inability, like animals, to photosynthesise. Additionally, the cell walls of fungi are composed by chitin instead of the cellulose found in plants. In 1993 comparison of the sequences of 25 proteins from fungi, plants and animals implied that fungi are more related to animals than plants (Baldauf and Palmer, 1993). More recent bioinformatics research based on the gene ontology AmiGo database for homologous polypeptide sequences revealed that among 552 such sequences, 78 were shared between plants, animals and fungi, 72 sequences were shared only between fungi and animals and 58 sequences were shared between fungi and plants (Moore and Meskauskas, 2006). This comprehensive genome scale comparison supports the view that fungi are more related to animals.

As one of large groups of organisms, fungi have been studied for many years. Although the collection of mushrooms as food by humans may start from prehistoric times, the systemic research of fungi, known as mycology, started from 16th century after the microscope being invented (Ainsworth, 1976). Modern studies of fungi as a

model organism involve in “biochemistry, genetics, molecular biology, biotechnology, biodiversity and taxonomy” (Alexopoulos et al., 1996). As a primary decomposer in nature, fungi can break down complex organic compounds and reuse them as nutrients; this “recycling” involves many enzymes and proteins for metabolism, homologues of which widely exist through other kingdoms, giving scientists insights for research on higher organisms. Some fungi are capable of producing antimicrobials as their natural biological products and research into their development for medical benefits continues. As more and more genomes are sequenced, their comparison helps to explain the evolutionary relationship between species (Fitzpatrick et al., 2006; Galaga et al., 2005; Nierman et al., 2005). Highlights of current fungal researches include carbon and nitrogen metabolism, pH regulation, regulation of the cell cycle in model organisms of fungi including *Aspergillus nidulans*, *Neurospora crassa*, and *Saccharomyces cerevisiae*. In industry, besides food supply and processing (wine, baking powder, soy sauce etc), fungi are also used for producing antibiotics (penicillin), medicine (drugs for cancer therapy), chemicals and enzymes, which now help humans control insects infection and diseases defence (Whalley, 1996).

For their enormous importance in variety aspects, the fungi are no doubt one of the most important groups of organisms on the planet.

1.2 *Aspergillus nidulans*

Aspergillus nidulans, named by Winter in 1884, is one of many species of filamentous fungi in the phylum Ascomycota. It has been used as a model organism for studying genetics, eukaryotic cell biology, metabolism and pathogenesis in laboratory (Martinelli and Kinghorn, 1994). There are several advantages of *A. nidulans* for scientific research: First, it is inexpensive to maintain under the laboratory conditions and can be grown with a wide range of carbon, nitrogen, sulphur and phosphorus sources. Second, it is a quickly growing homothallic fungus which is self-fertile and able to form sexual spores as a result of meiosis thereby making the crossing of strains straightforward. Third, the whole genome information of *A. nidulans* has been sequenced and published. There are 9500 protein coding genes located all through eight chromosomes been predicted by establishment of 30 million base pairs (Galaga et al., 2005). Thus, its genes can be cloned and transformed back into the genome and then to study the expression *in vivo* (Nierman et al., 2005). Furthermore, the comparison of *Aspergillus* genome sequences provides insights into genome evolution and gene regulation (Machida and Gomi, 2010). For example *A. fumigatus*, a fungus that can cause death in leukaemia patients, is distantly related to *A. nidulans* and *A. oryzae*, as only 50 % of the *A. fumigatus* genome can be aligned with the other two genomes. On the contrary, *A. fumigatus* is closely related to *Neosartorya fischeri*. The comparison of these latter two species helps research on pathogenesis, the sexual cycle and many other biological functions common to both species (Nierman et al., 2005). Genome comparisons have also shown that mating type genes occur in homothallic species implying that they have a role in regulating sex in these species. The conserved α - domain of MAT-1 protein of *A. nidulans* shares 43, 52, 59, and 59 % identity to Ascomycetes: *Podospora*

anserine, *Gibberella fujikuroi*, *Pyrenopeziza brassicae* and *Tapesi yallundae* (Dyer et al., 2003).

1.2.1 Growth conditions for *A. nidulans*

In common with most of the fungi, *A. nidulans* can grow well only by given water, air, necessary sources as well as traces of metals (Martinelli and Kinghorn, 1994). It utilises a wide range of carbon (glucose, glycerol, ethanol, etc), nitrogen (glutamine, glutamate, ammonium, nitrate, nitrite, etc), sulphur and phosphorus sources. It can grow both on solid surfaces and in liquid environments. Wild type strains of *A. nidulans* are able to grow in a wide pH range from 3.5 to 9, not because most of enzymes can work under extreme pH but the organism has “an efficient pH homeostatic mechanism” (Caddick et al., 1986). The best temperature for growth is between 25°C and 37°C, but *A. nidulans* can grow at higher temperature 45°C and has the ability to recover from a 50°C heat shock (Filliner et al., 2001).

1.2.2 The life cycle of *A. nidulans*

A. nidulans grows as hyphae, long filamentous cells which form cylindrical, thread-like branching structures. The major vegetative part composed of hyphae is called mycelium. New mycelium can be grown from parent hyphae, asexual spores (conidia) and sexual spores (ascospores). *A. nidulans* life can reproduce through three growth cycles: the parasexual cycle, the sexual cycle and the asexual productive cycle (Fig 1.1).

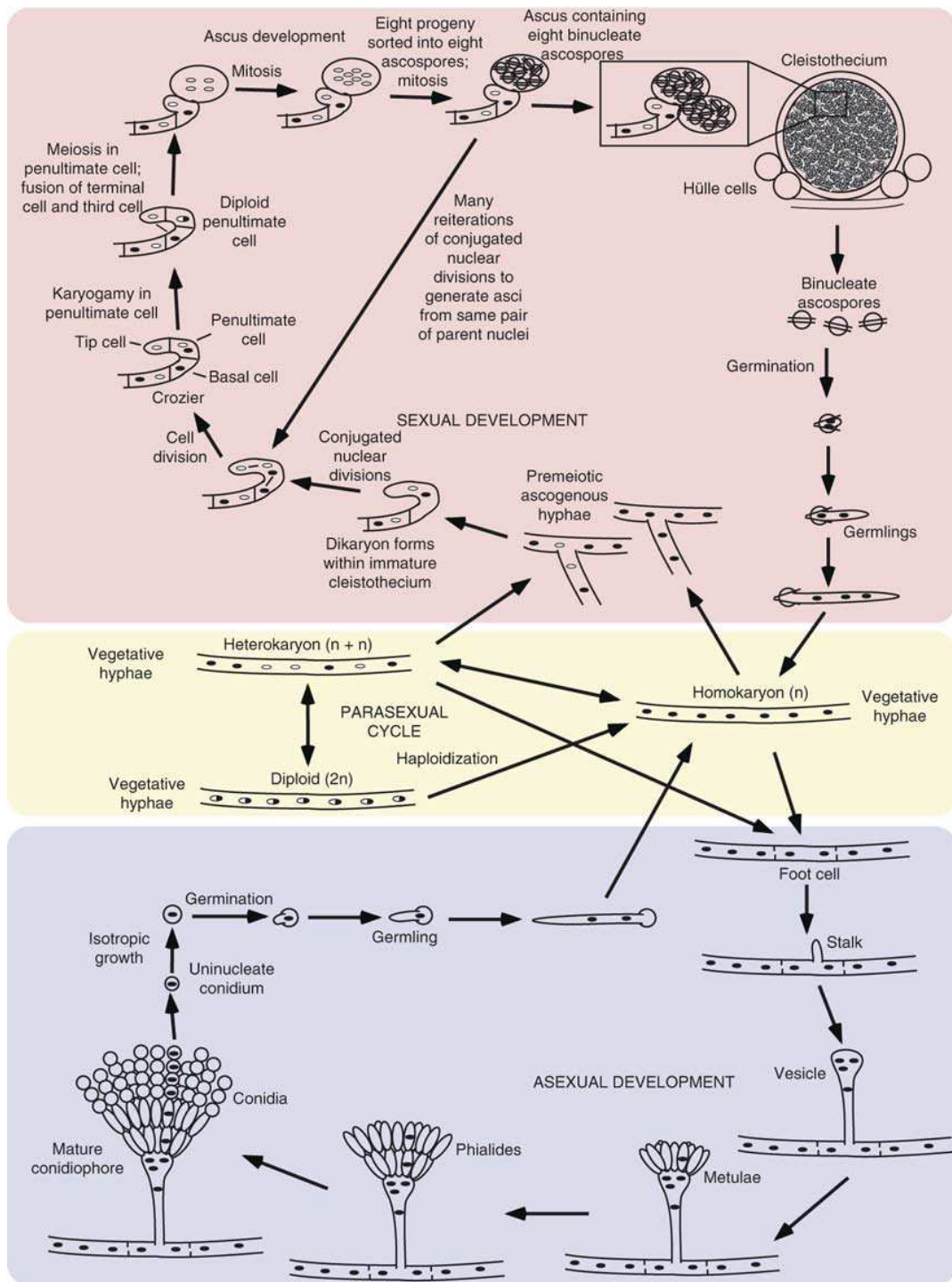


Fig 1.1: The life cycle of *A. nidulans*, taken from (Todd et al., 2007).

The asexual cycle begins when a single spore germinates to form tubular hyphae cells. The apparent specialisation of hyphae cells within the colony can be observed approximately 16 hours after the spore germination. The hyphae cell differentiates into a foot cell and forms a stalk, then a metulae by apical extension and then forming branches network of phialides to generate conidiophore (the asexual spores bearing structures). It takes 6-8 hours for initiation of conidiophore generation and formation of the conidia (Adams et al., 1998). After the first asexual cycle in the centre of the colony being completed within 24 hours, following outward asexual cycles take much less time. As the cell cycle in *A. nidulans* is about 100 minutes and there are dozens of conidia on each conidiophore, it is a quick and efficient way of asexual propagation. But, hyphae cells have to be exposed to air interfaces for conidiophore generation. Conidia are non-motile spores and generated through mitosis (also termed as mitospores). They are genetically identical to the parent hyphae cells and can develop into new organisms.

The initiation of sexual cycle could be considered as the termination of asexual cycle. It involves “the formation of multicellular fruiting bodies, called cleistothecia, that are surrounded by specialised cells termed Hülle cells” (Adams et al., 1998). The premeiotic hyphae form dikaryon cells within immature cleistothecium. After following nuclear division and cell division, it differentiates into basal cells, penultimate cells and tip cells. Haploid penultimate cells are formed by meiosis from dikaryon cells for further ascus development by several mitosis steps. Mature cleistothecium consists of hundreds of asci which containing eight binucleate ascospores. Each of these ascospores is ready for new germination. The sexual reproduction in *A. nidulans* can be performed homothallic, which requires no partner. A single spore generated homothallic colony is able to complete the sexual reproductive cycle without interaction

with other individual fungi which makes the strict control of genetic material available (Rossomando and Alexander, 1992).

The parasexual cycle is a genetic recombination without meiosis and it can occur at any time point through the whole life cycle. Normally it starts from the fusion of the hyphae of two strains containing two types of haploid nuclei to form a heterokaryon, which called as heterokaryosis. Limited crossing over takes place between homologous chromosomes of the diploid nuclei formed by the fusion of the unlike haploid nuclei. Finally, the vegetative haploids are formed by haploidisation from the diploid nuclei (Pontecorvo, 1956). This process is quite similar to meiosis and it is possible because the haploid and diploid forms are stable. However the diploid form is less stable than the haploid form and is prone to non-disjunction at mitosis leading to the production of highly unstable euploid and aneuploid nuclei. These euploid and aneuploid forms lose chromosomes at random to generate either the stable or diploid forms once more. As the parasexual cycle provides a rapid and strictly controlled way for mutation generation, DNA recombination between different species in the lab was developed for genetic manipulation (Clutterbuck, 1996).

1.3 Nitrogen metabolism in *A. nidulans*

Nitrogen is a major element and is found as many simple compounds and complicated complexes. It is also a fundamental element for all living organisms as it is found in proteins and nucleic acids. Thus, nitrogen metabolism is extremely important in life.

A. nidulans and *N. crassa*, as well as other fungi are able to utilise a wide array of nitrogen sources including ammonium, glutamine, glutamate, nitrate, nitrite, hypoxanthine etc. Utilisation of so many nitrogen sources requires the expression of

more than 100 genes and specific transcription regulation mechanisms of the metabolic pathways. Taking nitrate utilisation pathway for example, there are two structural genes of *niaD* and *niiA*, which encoding nitrate reductase (NiaD) and nitrite reductase (NiiA) to convert nitrate into ammonium (Tomsett and Cove, 1979). Besides that, there is one pathway-specific control gene *nirA* regulating the transcription levels of *niaD* and *niiA* and six additional genes known as *cnx* genes which encoding molybdenum cofactor interacting with nitrate reductase (Burger et al., 1991; Cove, 1979). Other pathways such as hypoxanthine utilisation also involve the structural gene, *hxA*, encoding purine hydroxylase and the regulatory gene, *hxB*, encoding an enzyme that works as a post-translational regulator or a cofactor for purine hydroxylases (Glatigny and Scazzocchio, 1995; Scazzocchio, 1994; Sealy-Lewis et al., 1979).

1.4 Nitrogen metabolite repression (NMR)

Although *A. nidulans* can utilise a wide array of nitrogen sources it uses ammonium or glutamine as its preferred source. When ammonium or glutamine is present in the growth medium the enzymes and permeases necessary for the utilisation of alternative nitrogen sources are absent. In the absence of the preferred nitrogen sources the presence of alternative nitrogen sources leads to the production of the enzymes and permeases necessary for their utilisation. A good example of this is the utilisation of nitrate described above. However when a preferred nitrogen source is present as a mixture with alternative nitrogen sources the enzymes and permeases necessary for utilisation of the alternative nitrogen sources are absent. This phenomenon is known as nitrogen metabolite repression (NMR) and demonstrates that *A. nidulans* has a signal transduction pathway that is able to recognise the presence of preferred nitrogen sources (Wilson and Arst, 1998). Ammonium and glutamine are designated primary nitrogen

sources while alternative nitrogen sources are designated secondary nitrogen sources. Metabolic pathways specific for the utilisation of secondary nitrogen sources that have the potential to be activated by the presence of the appropriate nitrogen sources but remain inactive (or repressed) in the presence of preferred nitrogen sources are termed as repressible activities. Thus, media containing a primary nitrogen source (ammonium or glutamine) are defined as nitrogen repressing conditions and conditions with only secondary nitrogen sources at present as derepressing conditions.

The first research in nitrogen metabolite repression in *A. nidulans* was performed with the isolation of mutants resistant to the toxic ammonium analogue methylammonium (*mea^R*) (Arst and Cove, 1969). Those mutants demonstrated the depression of repressible enzyme activities at the presence of ammonium. Mutants of two unlinked gene designated *meaA* and *meaB* showed derepression of ammonium repressible activities and *meaA* mutants fail to transport ammonium and methylammonium into the cell in a methylammonium-¹⁴C transport study (Arst and Page, 1973). Other mutants that lose the functions related to nitrogen metabolite repression were found and mapped to a single locus, designated *areA* which was believed to work as a principle regulatory gene. Two allelic *areA* mutants involved in nitrogen metabolite repression along with *creA* mutants involved in carbon catabolite repression were isolated (Arst and Cove, 1973). Since then, nitrogen metabolite repression has been widely researched and mutants were generated for study the role of *areA* gene in nitrogen metabolite repression and relatively interacted proteins and enzymes in *A. nidulans* as well as other representative fungi.

1.5 Regulation of nitrogen metabolite repression in *N. crassa*

Research into nitrogen metabolite repression has progressed in *N. crassa* which is another model organism related to *A. nidulans*. Research with these two model organisms has been complementary. For example, mutational analysis of nitrogen metabolite repression in *N. crassa* was established after the *areA* mutants of *A. nidulans* had been isolated (Coddington, 1976). However, the negative regulatory gene *nmrA* was isolated by searching the expressed sequence tag database of *A. nidulans* with its homologous gene sequence (*nmr-1*) from *N. crassa* (Andrianopoulos et al., 1998).

The first mutant resistant to methylammonium (*mea-1*) that was identified in *N. crassa* also showed derepression of ammonium repressible activities (Dunn-Coleman et al., 1984). Further mutational analysis identified five *nit* genes: a structural gene *nit-3* encoding nitrate reductase and the *nit-1* gene that was required for the production of the enzyme; the *nit-4* and the *nit-5* as specific regulatory genes in nitrate metabolite pathway; *nit-2* as a general regulatory gene (Coddington, 1976). Further researches in the *nit-2* mutant showed that it was unable to transport secondary nitrogen sources and induce the necessary utilisation pathways (Facklam and Marzluf, 1978; Fu and Marzluf, 1987a; Pendyala and Wellman, 1978). All these results suggest *nit-2* works as a global regulatory gene with its protein product controlling the regulation of the pathways from secondary nitrogen sources in a manner similar to the *areA* gene in *A. nidulans*.

The *nit-2* gene encodes a zinc-finger containing protein composed of 1036 amino acids which belongs to GATA family (Fu and Marzluf, 1990). The Nit2 protein is required for the stimulation of transcription of *nit-3* gene and many other structural genes related

in other nitrogen metabolite pathways (Fu and Marzluf, 1987b; Lee et al., 1990). It works with pathway-specific controlling proteins and binds to the GATA sites in the promoters of these structural genes to activate their transcription. Many genes directly involved in nitrogen metabolism are unable to express and thus lose their biological functions in *nit-2* mutants. Taking nitrate metabolism for example, both the global regulator Nit2 and the pathway-specific regulator Nit4 have to bind to the recognition sites of *nit-3* gene to activate its expression. The interaction between Nit2 and Nit4 is essential for the activation (Feng and Marzluf, 1998). The transcription level of *nit-2* gene is not self-regulated but related to different nitrogen conditions. It increases approximately three fold when growing under derepressing condition to repressing condition (Fu and Marzluf, 1987a).

The other major controlling gene is *nmr-1*, mutational analysis of which suggested it worked as a negative regulator (Debusk and Ogilvie, 1984; Dunn-Coleman et al., 1981; Fu and Marzluf, 1988). The encoded Nmr1 protein has been proved to form a specific protein-protein interaction with the C-terminal portion of Nit2. In the yeast two hybrid studies, Nit2 and Nmr1 were fused to GAL4 DNA binding domain and GAL4 activation domain separately. The *in vivo* specific binding between these two proteins activates the expression of the downstream reporter gene, which is detected by β -galactosidase assay. Electrophoretic mobility shift assay (EMSA) studies have shown Nmr1 protein fused to the GST protein inhibits the binding of a fragment of Nit2 to its DNA target *in vitro*. These results established that Nmr1 directly interacts with Nit2 and prevents its binding to recognition sites of DNA for gene expression activation (Xiao et al., 1995). Different internal or C-terminal deletions were made from *nit-2* gene to produce truncated proteins, which maintain the function to activate gene expression. It was shown that most of these *nit-2* mutants can still repress the nitrate

reductase syntheses, but deletions of certain amino acid residues in the α -helix of the zinc finger in the DNA binding domain or the 12 C-terminal residues were unable to respond to nitrogen metabolite repression and bind to Nmr1. These results strongly suggest that direct interaction between Nit2 and Nmr1 was fundamental to the nitrogen metabolite repression which involved two regions of Nit2 protein: a single zinc finger DNA binding domain and the extreme C-terminal sequence (Pan et al., 1997).

1.6 Regulation of nitrogen metabolite repression in *A. nidulans*

1.6.1 Positive regulator: AreA

In *A. nidulans*, synthesis of all enzymes subject to nitrogen metabolite repression is under the control of the general positive regulatory gene, *areA* gene (Arst and Cove, 1973). The *areA* gene encodes a protein consisted of 876 amino acids, designated AreA. In the absence of the primary nitrogen sources ammonium and glutamine, the global transcription factor AreA protein interacts with other specific transcription factors. The expression of many structural genes required for specific metabolite pathways is activated to facilitate the utilisation of the secondary nitrogen sources. For example, when the fungus grows with nitrate as nitrogen source, AreA will interact with NirA, which is a pathway-specific transcription factor, to activate the transcription of enzymes and proteins that are needed to utilise nitrate (Rand et al., 1987).

1.6.2 Mutational analysis of *areA*

Two classes of *areA* mutants have been isolated (Arst and Cove, 1973). One class was loss-of-function mutants designated *areA^r*, which is unable to grow on most of other

nitrogen sources but primary nitrogen sources ammonium or glutamine. These mutants show a repressed phenomenon by their inability to utilise various secondary nitrogen sources, and are known as repressed mutants. Some *areA* repressed mutants have temperature sensitivities. Another rare class of derepressed mutants, designated *areA^d*, is shown to be derepressed as the pathways for secondary nitrogen sources can be activated under growth conditions containing primary and secondary nitrogen sources. One representative mutant is *xprD-1* which leads to derepression of extracellular protease (Cohan, 1972). For example, when the mutant is growing on the medium containing repressing nitrogen sources ammonium or glutamine as well as powdered skimmed milk, the activity of synthesising extracellular protease is derepressed and the organism will digest the milk in the media. Thus the milk clearing around the colony on the plates can be used as a diagnostic test for the presence of derepressed activities. Different *areA^d* mutants display a spectrum in the extent of derepression for different repressible activities.

1.6.3 Structure of AreA and the GATA family

Analogous to Nit2, AreA is also a member of the GATA family of transcription factors (GATA TFs). All GATA proteins contain one or two highly conserved type IV zinc finger containing DNA binding domain (DBD). “The core of the zinc finger DNA binding domain is composed of two irregular antiparallel β -sheets and an α -helix, followed by a long loop” (Omichinski et al., 1993). The zinc ion is coordinated by four cysteines. It is the zinc finger motif and loop connecting with the two β -sheets that bind in the major groove of the target DNA while the following C-terminal tail for specific binding determination of target genes (Fig 1.2). GATA family is a group of evolutionally conserved proteins through vertebrate, invertebrate, plants and fungi.

The single finger in Nit2 and AreA protein is most closely similar to the C-terminal zinc finger of the vertebrate proteins (Kudla et al., 1990).

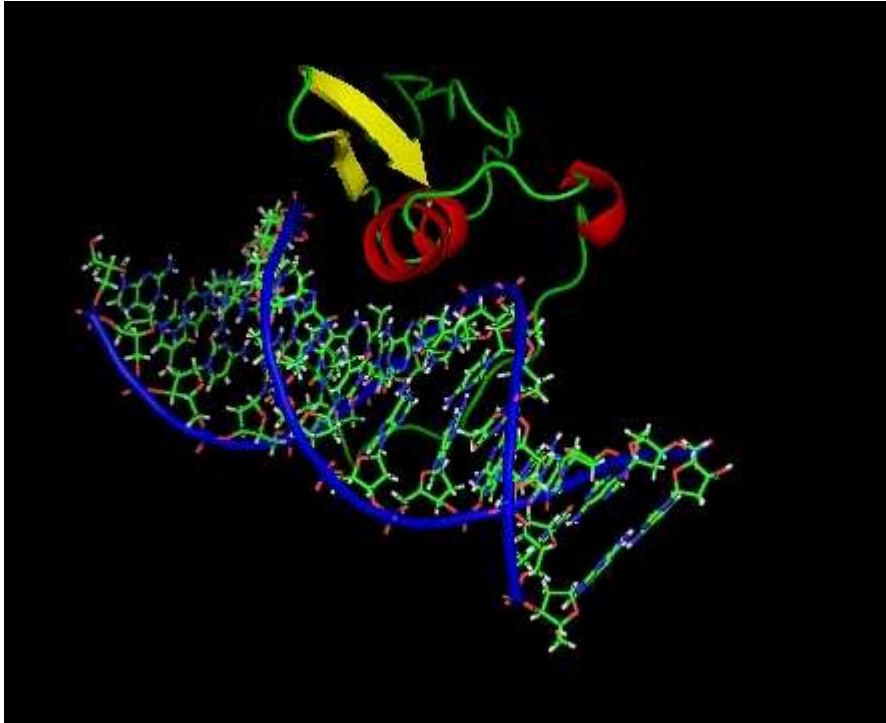


Fig 1. 2: Ribbon diagram of wild type DNA binding domain of AreA complexed to a 13 bp DNA containing a CGATA site.

Diagram is created by the PyMOL Molecular Graphics System (2002) of file (5gat. pdb) from RCSB Protein Data Bank. The α -helix (red) and two β -sheets (yellow) bind in the major groove of DNA (blue).

There are three types of GATA motifs in fungi: type IVa, type IVb and type IVc while animals only have type IVa and plants have type IVb. Type IVa (CX₂CX₁₇CX₂C) and IVb (CX₂CX₁₈CX₂C) have different spaces between two cysteine pairs and type IVc is a rare group of 19- to 21-residue motifs (CX₂₋₄CX₁₉₋₂₁CX₂C) (Fitzpatrick et al., 2006; Reyes et al., 2004; Teakle and Gilmartin, 1998). The diversity of GATA family in fungi suggests the possibility of multiple modes in evolution. As transcription factors, the GATA proteins not only regulate nitrogen metabolism (AreA, Nit2), but also work in the regulation of siderophore biosynthesis for iron uptake (SreA), regulation of expressions of light genes (WC-1, WC-2, SuB-1) and regulation of sexual development by controlling the formation of cleistothecia (NsdD) (Hass et al., 1999; Chen and Loros, 2009; Han et al., 2001).

AreA is also essential for chromatin remodelling. In the pathway for utilising nitrate, there is an intergenic region responsible for regulating the transcription of structural genes for nitrate and nitrite reductase (*niaA* and *niiA*). The intergenic region contains 4 binding sites for the specific regulator NirA and 10 GATA binding sites for the global regulator AreA (Punt et al., 1995). The intergenic region in the *niiA-niaD* complex promoter was fused to two proteins– a GST-AreA₆₆₃₋₈₀₉ fusion containing DNA binding domain and an untagged AreA₆₆₃₋₈₀₉. Mutants of individual and combinational GATA binding sites were then constructed and analysed by gel shift experiments. The results showed that AreA associated with four central GATA sites (5, 6, 7 and 8) of *niiA-niaD* complex contributing to transcriptional activation and the essential site 5 is occupied under both repressing and derepressing conditions. The structural investigation of *niiA-niaD* promoter by DNaseI and micrococcal nuclease (MNase) digestion revealed that under the derepressing condition, there is a dramatic reorganisation of nucleosomes. At least five nucleosomes -2, -2, +1, +2 and +4 besides the central GATA region are

lost as they showed sensitivity to MNase under derepressing condition. The same enzyme digestion experiments with *areA600* null mutants proved that AreA is totally responsible for this chromatin rearrangement (Muro-Pastor et al., 1999).

The Nit2 and AreA proteins contain highly conserved C-terminal tails that are also present in a wide array of other species. The 12 residues in C-terminal residues of Nit2 are thought to be essential for interacting with Nmr1 to mediate nitrogen metabolite repression (Pan et al., 1997). In contrast, the C-terminal 9 amino acids of AreA are not required *in vitro* for binding of NmrA to the C-terminal region of AreA (residues 662-876) that contains the zinc finger (Lamb et al., 2004).

1.6.4 Regulation of AreA

Gene expression can be controlled at many different levels. These regulatory mechanisms include transcription, mRNA stability, translation, posttranslational modification and direct protein-protein interactions. One of the regulating mechanisms has been shown to be the transcript stability of *areA* mediated through its 3'-untranslated region (3'-UTR). This was demonstrated by assessing the half-life of the *areA* transcript under repressing and derepressing conditions (Platt et al., 1996). It was found that the half-life of *areA*-mRNA was approximately 7 minutes under nitrogen repressing conditions, compared to 40 minutes under derepressing conditions, an increase of about 5 fold. However, the half-life of specific mRNA of 3'-UTR deleted *areA* gene remained the same under repressing conditions (22 minutes) and derepressing conditions (25 minutes). The presence of the *areA* 3'-UTR was found to accelerate degradation of the poly (A) tail, which decreases the transcript stability in response to the presence of ammonium or glutamine. In other words, the degradation

of the AreA transcript was initiated under repressing conditions by the presence of ammonium or glutamine. Post-translational modulation of transcription activating proteins is also a common way to regulate gene expression. One region possibly involved in post-translational modulation is the C-terminus adjacent to the DNA binding domain of *areA* gene. C-terminally deleted forms still maintain their transcription activating function but are unable to sense nitrogen metabolite repression conditions. This suggests that the C-terminus of this protein may have a nitrogen repression signal sensing site. It was thought that this site could potentially bind to the nitrogen signal factor such as ammonium or glutamine, or recognise some other sensing proteins to respond to the nitrogen conditions (Marzluf, 1997). Besides C-terminal tails, posttranscriptional modulation of AreA and Nit2 also involves another region, the zinc finger motif contained DNA binding domain. In *N. crassa*, changes to the residues in the α -helix region of the zinc finger DNA binding domain caused the Nit2 protein to activate expression of the nitrate reductase gene but was largely insensitive to nitrogen metabolite repression. This suggested that there may be a nitrogen repression sensing site in this domain (Pan et al., 1997). The mutational analysis of DNA binding domain of *areA* gene in *A. nidulans* showed that some residues in the extended loop structure were also essential for inducing functional AreA products (Platt et al., 1996).

1.6.5 Transcriptional repressor: NmrA

Nmr1 has been proved to bind directly to Nit2 to exert its inhibitory function in *N. crassa*. By searching *A. nidulans* expressed sequence tag (EST) database with *nmr-1* gene sequence the *nmrA* gene was identified and then isolated. It encodes a protein of 352 amino acids, which share approximately 60% sequence identity with the highly conserved C-terminus of Nmr1. Deletion of *nmrA* gene results in partial derepression

of activities involved in nitrogen metabolite repression. This implied that NmrA acts as a negative transcription regulator (Andrianopoulos et al., 1998). It was found that NmrA has a similar structure (see Fig 1.3) to the short chain dehydrogenase / reductase (SDR) family and it may have a function in redox sensing as it preferring binding to oxidised dinucleotides compared to the reduced dinucleotides (Lamb et al., 2003; Stammers et al., 2001). There are at least two characteristic motifs through the whole SDR family. One is a GlyXXGlyXXGly motif required for the nucleotide binding and the other is a triad of Ser, Tyr and Lys residues with catalytic properties (Filling et al., 2002). The structural studies showed that the nucleotide binding motif in NmrA was replaced by the sequence AsnXXGlyXXAla. The key catalytic amino acid tyrosine in the triad motif was replaced by a methionine residue. The replacement of the Tyr by Met would suggest that NmrA cannot be an active dehydrogenase of the SDR family. There is an essential dinucleotide (Rossmann) fold within a two-domain structure at the N-terminal region of NmrA. The N-terminal domain is composed of several parallel β -sheets connecting with α -helices and the C-terminal domain only has an α -helix and a β -sheet. The Rossmann fold is required for maintaining full biological function as well as the C-terminal domain of NmrA. A largely disordered loop region within residues 283-316 is located between C-terminal α -helix 9 and α -helix 10 (Stammers et al., 2001).

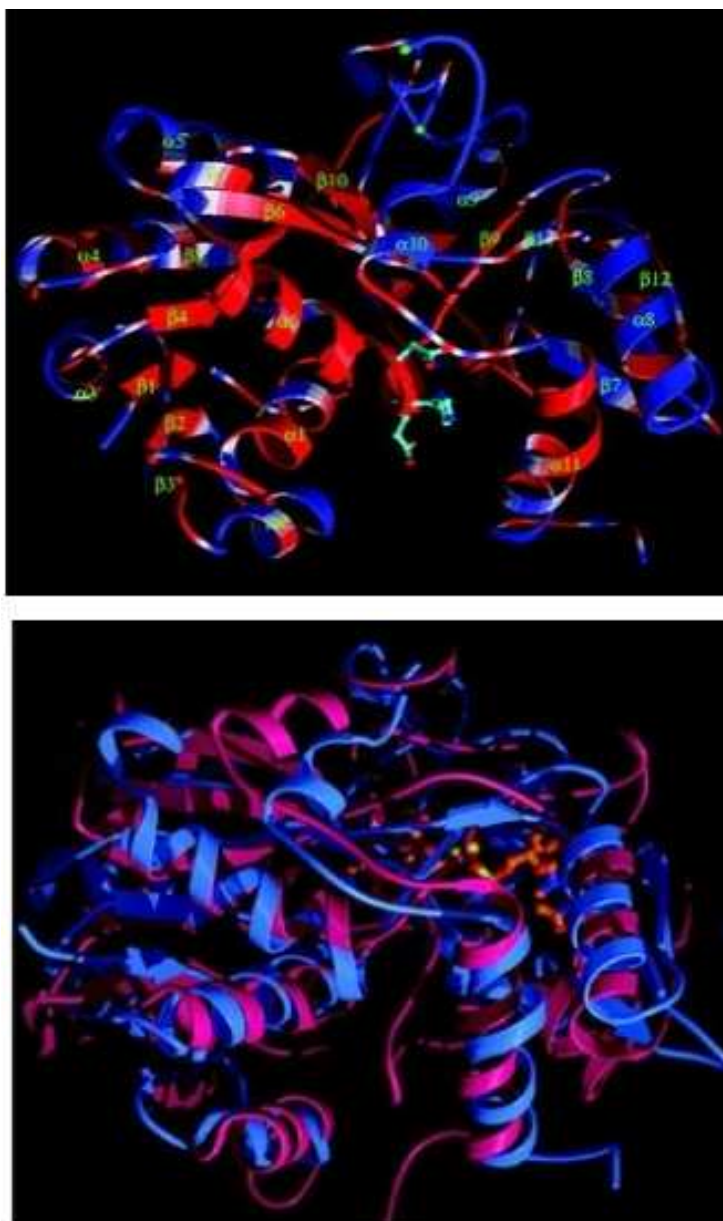


Fig 1. 3: Ribbon stereodiagrams of NmrA protein from (Stammers et al., 2001).

Upper: Overall secondary structure of NmrA protein. A dinucleotide (Rossmann) fold with a central core of parallel β -sheets (β 1- β 6) interconnected by α -helices (α 1- α 5), is located in the N-terminal region. The C-terminal region includes several β -sheets (β 7- β 12) and α -helices (α 6- α 11) and a disordered loop region positioned between α 9 and α 10. The conserved residues among the NmrA and Nmr1 are coloured red while the non-conserved residues are coloured blue.

Lower: Comparison of overall structure of NmrA (blue) and another SDR family member-UDP-galactose 4-epimerase (purple). The bound NAD^+ and UDP-glucose are shown as ball and stick representation.

1.6.6 Interaction of AreA and NmrA

According to the Nmr1 and Nit2 binding model in *N. crassa*, it was originally predicted that in *A. nidulans*, NmrA would exert its repressing effect on AreA by forming a binary complex and preventing its interaction with target genes. Isothermal titration calorimetry (ITC) using native full length NmrA and a His-tagged C-terminal fragment of AreA (AreA₆₆₂₋₈₇₆) demonstrated that the two interacted with a 1:1 stoichiometry and K_D of 0.26 μ M (Lamb et al., 2003). The crystal structural analysis showed that α -helices 1, 6 and 11 of NmrA (352 residues) were involved in the interaction with a zinc finger of AreA (residues 663-727, AreA Zf) (Kotaka et al., 2008). The C-terminal tail (residues 702-715) of AreA Zf interacts with α -helices 1, 6 of NmrA composed by the hydrogen bond between Ser₇₁₁ and Glu₁₉₃. Another interaction region between AreA Zf and NmrA involves hydrophobic bonds between two parallel α -helices from each protein and a single hydrogen bound between Asn₆₉₅ and Ser₃₂₆. The structural analysis also showed that the binding region of GATA DNA on AreA Zf is partially overlapped by NmrA which suggests that NmrA competes for the same binding site as DNA as proposed by the model in *N. crassa*. However, the ITC experiments showed that the AreA Zf binds to the GATA-containing DNA with NmrA at the present *in vitro* which is contrary to the structure information. Moreover, the deletion of the C-terminal 9 AreA amino acids does not reduce the affinity of AreA for NmrA, unlike the situation in *N. crassa* while the C-terminal 12 amino acids of Nit2 are required for Nmr1 binding (Lamb et al., 2004). This implies that *in vivo* NmrA might need a cofactor to prevent full length AreA from binding to its target proteins or possibly exert its repression on transcription by an indirect way, such as controlling the entry rate of AreA into the nucleus.

At the time the research reported in this thesis began, work from a previous PhD student (Johnson, 2008) revealed that transformants overproducing wild type NmrA *in vivo* showed a growth inhibited phenotype on media containing single secondary nitrogen sources- a phenotype similar to that of a strain deleted for the *areA* gene. Based on these observations, a hypothetical model involving an indirect effect of NmrA was proposed. At wild type levels, NmrA protein traps AreA in a binary complex in the cell, thus preventing AreA entry into nucleus. Under inducing conditions, the increased stability of the AreA mRNA allows AreA protein levels to build up to a threshold at which they begin to escape from the NmrA repressor and enter the nucleus. Once in the nucleus AreA is free to initiate the transcription of genes required to utilise the given nitrogen source, as well as auto-regulate its own expression. The model predicts that extensive overproduction of NmrA in a cell would result in a decrease in the free pool of AreA. Even under inducing conditions, while the AreA protein level is at its highest level, the majority would be captured by NmrA protein. This would reduce the rate of entry of AreA into the nucleus inhibiting the initiation of the transcription of those genes involved in utilising secondary nitrogen sources. Low levels of expression of permeases and enzymes needed in the secondary metabolite pathway would lead to an inhibited growth phenotype.

However, the recent research revealed that the overproduction of NmrA did not prevent accumulation of AreA in the nucleus under nitrogen starvation conditions (Wong et al., 2007). Transformants overproducing a FLAG epitope tagged NmrA (NmrA^{FLAG}) were constructed in a strain that was producing AreA tagged with a hemagglutinin epitope, AreA^{HA}. Immunostaining analysis revealed that AreA^{HA} accumulated in the nucleus under nitrogen starvation conditions with or without overproduction of NmrA. Reverse PCR (RT-PCR) experiments showed that the mRNA levels of NmrA and AreA

were inversely related and varied depending on the nitrogen status of the growth medium. For example, *areA*-specific mRNA is lower under repressing conditions whereas *nmrA*-specific mRNA is higher under these conditions. Under derepressing conditions (e.g. growth on nitrate) *areA*-specific mRNA is higher and *nmrA*-specific mRNA is lower. Furthermore the same study revealed that the function of AreA was prevented by the overproduction of NmrA and that the expression of *nmrA* gene was activated by the bZIP transcription protein MeaB (Wong et al., 2007).

1.6.7 TamA-the co-activator?

TamA mutants were first isolated on the basis of their resistance to the toxic nitrogen analogues thiourea, aspartate hydroxamate, chlorate and methylammonium (Kinghorn and Pateman, 1975). These mutants (*tamA*^r) showed not only partially repressed ammonium regulating activities similar to some *areA* mutants (*areA*^r), but also had lower levels of NADP-glutamate dehydrogenase (NADP-GDH) activity compared to the wild type level in *areA*^r mutants. After the isolation of the *tamA*^r50 mutant, which is unable to utilise other nitrogen sources than ammonium, and the derepressed *tamA*^{d1} mutant, TamA was proposed to work as a global regulator involved in mediating nitrogen metabolite repression (Pateman and Kinghorn, 1977). However, others later failed to isolate the repressed and derepressed mutants from the same selection protocol and instead proposed that the derepressed phenotype of *tamA*^{d1} was due to *glnA* mutation selected from partially repressed *tamA* mutant background (Arst et al., 1982). They proposed that the repressed and derepressed phenotypes were combination of mutations of other genes (*pryob* involved in vitamin B₆ biosynthesis) with *tamA*. The isolation of null *tamA* mutants supported this hypothesis as it had a partially repressed phenotype (Davis et al., 1996).

Although mutational analysis of the *tamA* gene did not support TamA as a major regulator similar to AreA, a regulatory function in nitrogen metabolite repression cannot be ruled out. For example, the *tamA24* mutants result in the reduction of the expression of acetamidase structural gene *amdS* in background of *areA102* mutants (strong growth on acrylamide medium) whilst having no effect in the background of the wild type *areA* strain (Davis et al., 1996). This result suggested TamA may work as a co-activator with AreA protein. Fusion of TamA to DNA binding domain of reporter genes (*facB*, acetate regulatory gene; *amdR*, regulatory gene involved in acetamide, omega amino acid and lactam metabolism) also showed TamA helped to increase the gene expression and the activities of these fusion proteins were dependent on AreA protein (Small et al., 1999).

Sequence analysis of TamA demonstrated it was composed of 739 amino acids, similar to Uga35p/Dal81p/DurLp protein of *S. cerevisiae* between residues 391-402 and residues 543-552. They both contain a Zn(II)₂Cys₆ DNA binding domain which is conserved through many regulators in fungi. However, mutational analysis revealed that this zinc finger motif was not required for the function of TamA (Davis et al., 1996). But later research of a series of *tamA* mutants with different sequence changes suggested that the overall conformation of TamA was essential for the protein function (Small et al., 2001). Further mutational researches showed that the C-terminal region (residues 865-876) of AreA were essential to the binding of TamA to FacB protein while the N-terminal amino acids 44-218 also contributing to the interaction. It was proposed that NmrA bound to the AreA Zf domain and the extreme AreA C-terminus and that the C-terminal 58 amino acids of AreA were required for the binding of TamA. TamA may recruit additional AreA molecules to the promoter region to form TamA-AreA complex or that it displace the NmrA from the NmrA-AreA complex to

activate transcription (Small et al., 1999). Comparison of the structures of an AreA Zf - DNA complex with an AreA Zf - NmrA complex showed that there was a difference in the orientation of the C-terminal tail of AreA. This observation raises the possibility that the interaction of TamA with the C-terminal region of AreA could change the conformation of the complex (Kotaka et al., 2008). The possibility of the third protein interacting with TamA and AreA and modulating their activation function cannot be ruled out. In this regard, it has been shown that TamA interacts with AreA and a third protein, LeuB, to activate expression of the *gdhA* gene encoding NADP-linked glutamate dehydrogenase (NADP-GDH) (Polotnianka et al., 2004).

In summary, TamA is thought to be a co-activator with AreA involved in mediating nitrogen metabolite repression; however the exact mechanisms remain unclear.

1.7 Regulation of nitrogen catabolite repression in yeast *S. cerevisiae*

S. cerevisiae is a yeast that belongs to a different lineage from *A. nidulans* and *N. crassa* within the Ascomycota and has been used for many years as a model system for research into metabolism (Fitzpatrick et al., 2006). *S. cerevisiae* regulates its use of nitrogen sources through a system called nitrogen catabolite repression (NCR) and like *A. nidulans* and *N. crassa*, utilises glutamine as its preferred nitrogen source.

Unlike the single major regulator AreA or Nit2 present in *A. nidulans* or *N. crassa*, respectively, *S. cerevisiae* has two positive regulators, Gln3 and Gat1. Mutants of the *gln3* gene were first isolated under conditions of glutamine limitation. The glutamine synthetase (*gln1*) is reversibly inactivated or repressed when transferred from glutamate to glutamine as nitrogen source (Legrain et al., 1982). The *gln3* mutants were able to

produce glutamine synthetase and NAD-linked glutamate dehydrogenase when grown with glutamate. Overproduction of Gln3 in transformants showed an elevated rate of the synthesis of the glutamine synthetase subunit compared to wild type when transferred from glutamine to glutamate as nitrogen source. This result suggested Gln3 was a positive regulator required for glutamine repressible activities (Mitchell and Magasanik, 1984). Sequence analysis revealed that Gln3 contained a putative zinc finger motif highly homologous to the single zinc finger of AreA and Nit2 in *A. nidulans* and *N. crassa*, respectively, which suggested Gln3 may bind to GATA sequence of target genes. Immunoprecipitation experiment showed that Gln3 bound to the GATAA sequences in the upstream activation region (UAR) of Gln1 (glutamine synthetase) as well as other sensitive promoters to activate transcription of genes controlled by nitrogen catabolite repression (Coffman et al., 1996; Minehart and Magasanik, 1991). The Gat1 (Nil1) was discovered to be responsible for mediating other Gln3-independent glutamine repressible regulation. For example, the general amino acid permease (*gap1*) was not detected when grown with glutamine. In *gln3* mutants, although the expression level of *gap1* was quite low when utilising glutamate as nitrogen source, the expression of *gap1* was much stronger when urea or ammonia were present in the medium (Stanbrough et al., 1995). Similar to Gln3, Gat1 has a conserved single zinc finger motif for recognition of the sequence GATAAG (Stanbrough and Magasanik, 1996).

Similar to the regulation of AreA and Nit2, Gln3 and Gat1 are also regulated by the nitrogen source status of the growth medium and through interaction with negatively acting repressors. Gln3 is activated by a decrease in the concentration of glutamine and Gat1 is activated by a decrease in the concentration of glutamate (Stanbrough et al., 1995). There is a negative regulator in *S. cerevisiae*, the Ure2 protein, which inhibits

the function of Gln3 protein. Glutamine synthetase is required for glutamine/glutamate metabolism and this enzyme is normally present at an increased level in the presence of glutamate and at a reduced level in the presence of glutamine. However, the increase of this enzyme is prevented in *gln3* mutants in the presence of glutamate, and high levels of the enzyme are found in *ure2* mutants in the presence of glutamine (Courchesne and Magasanik, 1988). These results indicated that *gln3* and *ure2* genes may have opposite functions in gene regulation. The *ure2* gene is localised on chromosome XIV, and encodes a protein composed of 354 amino acids, the sequence of which is homologous to glutathione S-transferases (Coschigano and Magasanik, 1991). The Ure2 protein has been shown to exert its inhibitory effect by binding to Gln3. But instead of inhibiting the binding of Gln3 to the GATA sequence of target genes, the interaction with Ure2 facilitates the nuclear exclusion of Gln3 under conditions of nitrogen sufficiency. This prevents Gln3 from binding to GATA sequences necessary for the initiation of gene expression (Beck and Hall, 1999; Kulkarni et al., 2001). In contrast, under nitrogen limitation condition, Gln3 and Gat1 accumulate in the nucleus and bind to the GATA sequences found in the promoter regions of structural genes involved in nitrogen catabolite repression.

There are other negative regulators involved in the regulation of nitrogen metabolism in *S. cerevisiae*. For example, negative regulators Dal80 and Deh1 are members of GATA family. The Dal80 protein was discovered by its inhibitory effect on the expression of a subset of genes responsive to nitrogen regulation. It is been shown that in *dal80* mutants, expression levels of many genes relating to nitrogen catabolite repression increase when proline is used as nitrogen source (Chisholem and Cooper, 1982). Deh1 was discovered as the homolog of Dal80 and negatively regulates some genes involved in nitrogen metabolism (Coffman and Cooper, 1997). Dal80 and Deh1

were reported to compete with the same GATA sequence binding sites of Gln3 and Gln1 in the target genes in response to nitrogen catabolite repression (Cunningham et al., 2000). Moreover, the derepressed activities observed in *ure2* mutants under nitrogen repressing conditions suggest there is another protein that complements the function of Ure2 protein (Coffman et al., 1994). It is been discovered that the major regulator Gcn4, which is involved in mediating the gene expression under amino acid starvation conditions is also involved in nitrogen catabolite repression by directly inhibiting the function of Gln3 under nitrogen repressing conditions (Natarajan et al., 2001; Sosa et al., 2003).

In summary, the nitrogen catabolite repression in *S. cerevisiae* has some similarities with nitrogen metabolite repression in *A. nidulans* and *N. crassa* as they all have positively acting transcription activators that contain a highly conserved GATA DNA binding domain. However, in these three species, there are differences in the regulatory mechanisms; the biggest of which is that the negative regulator Ure2 prevents the entry into the nucleus of the positive regulator Gln3 in *S. cerevisiae* but this does not appear to be the case in *A. nidulans* and *N. crassa* (Wong et al. 2007; Xiao et al., 1995).

1.8 Carbon catabolite repression (CCR) and its integration with nitrogen metabolite repression

A. nidulans can utilise most organic carbon sources for growth including glucose, glycerol, methanol, acetate and sucrose. For each carbon source there is a specific catabolic pathway for utilisation, and the genes encoding the necessary enzymes and permeases are themselves regulated at the level of transcription by the interplay of

positive and negative control proteins. For example, the *qut* gene cluster is required for the utilisation of quinate as a carbon source. The *qut* cluster comprises three structural genes *qutB* (dehydrogenase), *qutC* (dehydratase), *qutE* (catabolic type III-dehydroquinase); two regulatory genes, *qutA* (positive regulator) and *qutR* (negative regulator) and *qutD* (permease), *qutG* and *qutH* (unknown function) (Grant et al., 1988; Lamb et al., 1992). In the presence of quinate, the transcription levels of all these 8 genes are stimulated by the activator QutA and the three enzymes (QutB, QutC, QutE) convert quinate to protocatechuate (PAC) (Lamb et al., 1996; Park, 2004). Another example in the utilisation of ethanol, structural genes *alcA* and *aldA* encoding alcohol dehydrogenase I (ADHI) and aldehyde dehydrogenase (ALDH) are under the control of the pathway-specific activator (*alcR*) (Felenbok, 1991; Pateman et al., 1983).

In a manner analogous to the nitrogen source selection, there are also preferred carbon sources for utilisation in *A. nidulans*. Glucose and related sugars are preferred carbon sources and their presence in mixture with non-preferred sources inhibits the transcription of genes encoding the permeases and enzymes necessary for the catabolism of the non-preferred carbon sources. For example, in the presence of glucose and ethanol or glucose and quinate, the pathways for utilising ethanol and quinate remain inactive. This process is called carbon catabolite repression (CCR). The phenomenon of carbon catabolite repression implies that there is a signal transduction pathway that operates at a genomic level to override pathway-specific controls.

Some nutrients can be utilised as both carbon and nitrogen source, for example proline and acetamide. The *prn* cluster involved in the pathway for proline utilisation consists of 5 genes, *prnA-D* (*prnA*, pathway-specific transcriptional regulatory gene; *prnB*,

proline permease gene; *prnC*, L- Δ^1 -pyrooline-5-carboxylate dehydrogenase gene; *prnD*, proline oxidase gene) and *prnX* (Gavrias, 1992; Jones et al., 1981). Ammonium or glucose alone reduces transcriptions of *prn* genes, but for full repression a mixture of repressing nitrogen and carbon sources must be present (Gavrias, 1992). This observation shows that the controls of nitrogen metabolite repression and carbon catabolite repression are integrated. Strains carrying an *areA* loss-of-function allele (*areA^r* mutants) are unable to utilise proline as a nitrogen source in the presence of a repressing carbon source (e.g. glucose) but can utilise proline in the presence of a non-repressing carbon source (Arst and Cove, 1973). This phenotype was exploited to select mutants altered in carbon catabolite repression in an *areA^r* mutant strain growing on medium containing proline as nitrogen source and glucose as carbon source (Arst and Cove, 1973). This selection facilitated the isolation of carbon catabolite repression derepressed mutants designated *creA^d*.

After the *creA* gene had been identified, three further genes, *creB*, *creC* and *creD* were specified to get involved in the control of carbon catabolite repression (Hynes, 1977; Hynes and Kelly, 1977; Kelly, 1980). Molecular analysis showed that the *creA* gene encoded a DNA binding protein that had a Cys₂-His₂ zinc finger that binds to a 5'-(G/C)(A/T)GG(G/A)G-3' sequence (Dowzer and Kelly, 1991; Kulmberg et al., 1993). The CreA protein works as a general negative repressor regulating carbon catabolic pathways subject to carbon catabolite repression. For example, in the presence of glucose, the CreA protein binds to the promoters of the specific positive regulatory gene *alcR* and the structural genes *alcA* and *aldA* in the pathways for utilising ethanol (Lockington et al., 1987; Mathieu and Felenbok, 1994). The regulation on CreA is not clearly understood, however, it is known that autoregulation of *creA* transcription is not required for CreA-mediated repression and that large scale

degradation or regulated cellular localisation are similarly not required (Roy et al., 2008). Analysis of *creB* and *creC* mutants showed that in the presence of glucose, the expressions of enzymes for the utilisation of ethanol and acetamide were derepressed. Furthermore, these mutants have greatly reduced activities of enzymes for utilising D-quinic acid and L-proline compared to the wild type strain when they are used as sole carbon sources (Hynes and Kelly, 1977). The CreB protein contains 6 deubiquitination homology domains and is a member of a deubiquitinating protease family (Lockington and Kelly, 2001). The CreA protein is proposed to be a substrate for CreB to form a binding complex *in vivo* which suggests the CreA may undergo the deubiquitination regulation (Niyom, 2008). The *creC* gene encodes a protein composed of a proline-rich region, a putative nuclear localisation region and five WD40 repeats (Todd et al., 2000). It has been showed that CreB and CreC form a deubiquitination complex *in vivo* to modify or stabilise ubiquitinated proteins (Lockington and Kelly, 2002). The observation of *creD34* mutant showed it suppressed the phenotypes of *creC* and *creB* mutants. Thus, CreD was characterised as a repressor working in the ubiquitination relating both to CreB and CreC (Boase and Kelly, 2004).

Further molecular analysis of the *prn* cluster identified a positive *cis*-acting element (designated ADA) within the divergent *prnD-prnB* promoter. The CreA protein is proposed to repress the activity of the ADA element (Gonzalez et al., 1997). The presence of this element explains why an *areA* loss-of-function mutant is able to utilise proline as a nitrogen source in the absence of a repressing carbon source. This is because under carbon repressing conditions the CreA protein will inhibit the positive action of the ADA element; therefore none of the *prn* genes will be transcribed. Under carbon non-repressing conditions the positively acting ADA element will function and

facilitate transcription of the *prn* cluster even though a functional AreA protein is lacking.

In some experiments it is important to be able to decouple genes normally subject to control by nitrogen metabolite repression from this level of regulation. For example, experiments described later in this thesis required the expression of the *nmrA* gene to be decoupled from nitrogen metabolite repression and this was achieved by placing the *nmrA* gene under the control of the quinate inducible *qutE* promoter. The *qutE* promoter in wild type strains is inducible by the presence of quinate and is also subject to carbon catabolite repression which controls the transcription of the *qutE* gene that encodes a type III-dehydroquinase. Therefore, transcription of *nmrA* gene from the *qutE* promoter can be switched on or off by the use of either quinate or glucose as carbon source without regard to the nitrogen source.

1.9 Regulation by proteolysis

Site-specific proteolysis causing the degradation of control proteins has been recognised as an important posttranslational regulation mechanism. It is been extensively investigated in many cells and organisms. In the envelope stress response in *E.coli*, the signal transduction pathway is composed of a proteolytic cascade for the degradation of RseA (a membrane-spanning anti- σ factor) and this degradation releases the σ^E factor from the inhibitory complex with RseA. This facilitates RNA polymerase to transcribe downstream genes for protein synthesis (Chaba et al., 2007). The homeodomain-interacting protein kinase 2 (HIPK2), a regulator conserved from flies to human, mediates degradation of other proteins involved in cell growth, development and apoptosis. HIPK2 is itself degraded by the ubiquitin-proteasome

system (UPS) (Sombroek and Hofmann, 2009). The degradation of the negatively acting Aux/IAA protein by an ubiquitin-protein ligase, releases free ARFs (auxin response factor) for gene transcription in auxin signalling through all plants (Rogg and Bartel, 2001). The examples described above show that proteolytic processing of proteins involved in a wide array of metabolic and developmental pathways is an important and widespread method of regulation.

Proteins enriched in proline (P), glutamic acid (E), serine (S) and threonine (T), so called PEST proteins are targets for proteolysis (Rechsteiner and Rogers, 1996). One example in *A. nidulans* is the CreB protein involved in carbon metabolite repression which has four PEST sequence acting as proteolytic signal targets (Lockington and Kelly, 2001; Niyom, 2008). There are other proteins in *A. nidulans* containing one or multiple PEST regions. RosA, a negative regulator of sexual development was reported to have two putative PEST sequences (Vienken et al., 2005). VeA protein (coded by velvet gene *veA*) which has one PEST sequence is involved in sexual development, regulation of secondary metabolism and light response (Kato et al., 2003; Kim et al., 2002; Purschwitz et al., 2008).

A well understood example of proteolytic processing is provided by the activation of an *A. nidulans* transcription activator in response to a change in the ambient pH. PacC is a pH-dependent zinc finger containing regulator required in wild type strains for activating alkaline-expressed genes such as *ipnA*, an isopenicillin N synthase gene and inhibiting acid-expressed genes such as *gabA*, a γ -aminobutyrate (GABA) permease gene (Espeso and Arst, 2000; Espeso and Penalva, 1996; Espeso et al., 1997). The full length of PacC is 72 kDa and undergoes a two-step proteolysis in response to alkaline ambient pH. The first proteolytic event removes an inhibitory C-terminal domain that

renders the protein sensitive to a second proteolytic event that yields the active protein (Diez et al., 2002; Hervas-Aguilar et al., 2007a; Hervas-Aguilar et al., 2007b).

In summary, proteolysis as a regulation control is quite common through living organisms and while most of them involved in the response of exogenously supplied stimuli, there are others form part of normal programs in cell cycle progression.

1.10 Aims of the study

In *A. nidulans*, the involvement and interplay between AreA and NmrA in the regulation of nitrogen metabolite repression has been previously investigated. The regulation of AreA has been shown to involve multiple layers: transcription initiation, autoregulation, mRNA stability and posttranslational modulation by direct protein-protein interactions. The functions of both the AreA and NmrA proteins have been extensively studied and mechanism of activation of pathways of utilising non-preferred nitrogen sources have been well characterised.

At the time the research reported in this thesis was begun there was no information available regarding the *in vivo* effects of NmrA overproduction or the possible role of proteolysis in the regulation of nitrogen metabolite repression. The aims of the project were therefore to characterise the *in vivo* effects of the overproduction of NmrA and to investigate the possible role of proteolysis in nitrogen metabolite repression.

Chapter 2 Material and Methods

2.1 Chemicals and sources

All chemicals and reagents were supplied by BDH, unless otherwise stated, and were of AnalaR or equally high purity. Growth media was supplied by Difco. Agarose TOPO TA Cloning kit, Uera and *Enzcheck* Protease assay kit were obtained from Invitrogen. Plasmid purification kits were purchased from Qiagen. Origami™ 2 competent cell set was from Novagen. PfuUltra™ II Fusion HS DNA Polymerase was obtained from STRATAGENE. NAD⁺, ampicillin, Coomassie Brilliant Blue R250, bovine serum albumin, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), vitamins were all supplied by Sigma. Nitrocellulose and filter paper discs were supplied by Whatman and Bio-Rad provided blotting pads. Full range Rainbow Molecular Weight Markers and Hyperfilm ECL were obtained from Amersham Biosciences.

2.2 Media and supplementation

2.2.1 Luria Broth (LB)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
dH ₂ O to	1 litre
Adding 1.5 % (w/v) agar for Luria broth agar (LA) before autoclaving.	

2.2.2 Antibiotic solutions

Antibiotic	Stock concentration	Final concentration
Ampicillin	50 mg mg ⁻¹ (in dH ₂ O)	100 µg mg ⁻¹
Chloramphenical	70 mg mg ⁻¹ (in ethanol)	35 µg mg ⁻¹
Tetracycline	12.5 mg mg ⁻¹ (in ethanol)	12.5 µg mg ⁻¹
Streptomycin	12.5 mg mg ⁻¹ (in ethanol)	50 µg mg ⁻¹

2.2.3 *A. nidulans* Minimal Media (AMM)

A. nidulans minimal media (AMM) was prepared as the method described by Pontecorvo (Pontecorvo et al., 1953), and modified by Roberts (Roberts, 1963). 10 x AMM stock was diluted 10 times with dH₂O, by adding 1.8% (w/v) agar for solid media preparation. After the media had been autoclaved, 1 M MgSO₄ sterilised stock was added at a final concentration of 5 mM. Other appropriate supplements and nitrogen and carbon sources were added, too.

2.2.4 AMM stock salts (10 x)

KCl	5.2 g l ⁻¹
KH ₂ PO ₄	15.2 g l ⁻¹
*FeSO ₄ •7H ₂ O	10 mg l ⁻¹
*ZnSO ₄ •7H ₂ O	88 mg l ⁻¹
*CuSO ₄ •5H ₂ O	4 mg l ⁻¹
*MnSO ₄ •4H ₂ O	1.5 mg l ⁻¹
*NaB ₄ O ₇ •10H ₂ O	1 mg l ⁻¹
*(NH ₄) ₆ Mo ₇ O ₂₄ •10H ₂ O	0.5 mg l ⁻¹

*These salts were kept as a 1000 x stock, with 10 ml l⁻¹ used to make up the 10 x AMM stock salts. The pH of the 10 x AMM was adjusted to pH 6.5 by using 5 M NaOH.

2.2.5 Nitrogen sources

Nitrogen source	Stock solution	Final concentration
Ammonium tartrate	1 M	10 mM
Sodium nitrate	1 M	10 mM
Proline	1 M	10 mM
Hypothanxine	1 M	5 mM

2.2.6 Carbon sources

Carbon source	Stock solution	Final concentration
Glucose	40% (w/v)	0.4% (w/v)
Glycerol	50% (w/v)	0.4% (w/v)
Quinic acid	20% (w/v)	0.5% (w/v)

The quinic acid solution was made from free acid and adjusted to pH 6.5 using 5 M NaOH before autoclaving.

2.2.7 Media supplements

Supplement	Stock solution	Final concentration
Pyrodoxine	0.5 mg ml ⁻¹	0.5 µg ml ⁻¹
Para-amino benzoic acid	1 mg ml ⁻¹	1 µg ml ⁻¹

2.3 Buffers and solutions

2.3.1 DNA manipulation and analysis

Tris acetate-EDTA (TAE) electrophoresis buffer (50 x stock)

2 M Tris base
 1 M sodium acetate
 50 mM EDTA
 The solution was adjusted to pH 8.2 with acetic acid

Agarose gel loading buffer (10 x stock)

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Ficoll (type 400)	25% (w/v)

Acetate solution

NaAc	24.61 g
EDTA	0.37 g
dH ₂ O to	1 litre

The solution was adjusted to pH 7.0 with acetic acid, stored at 4 °C.

2.3.2 *E.coli* transformation buffers

1 M pH 6.5 MOPS (10 x stock)

MOPS	20.94 g
dH ₂ O to	100 ml
pH was adjusted to 6.5 with 5 M NaOH. Filter sterilised and stored at -20 °C.	

Calcium Chloride 0.5 M (10 x stock)

CaCl ₂	7.35 g
dH ₂ O to	100 ml

Autoclaved and stored at -20°C

pH 6.5 MOPS (1 x)

CaCl ₂ (10 x)	1 ml
pH 6.5 MOPS (10 x)	1 ml
dH ₂ O	8 ml

2.3.3 PCR reaction solution (50 µl)

Template DNA (100 ng)	1 µl of 100 ng µl ⁻¹ stock
10 x buffer	5 µl
PfuUltra TM II Fusion HS DNA Polymerase (µl)	1 µl
Primers (0.2 µM each)	1 µl + 1 µl of 10 µM stock
dNTPs (1 mM)	5 µl of 10 mM stock
dH ₂ O	36 µl

2.3.4 Protein solubility and purification buffers

Buffer 1	0.1 M Tris HCl pH 7.5, 1 mM DTT buffer
Buffer 2	50 mM potassium phosphate pH 7.2, 1 mM DTT, 150 mM NaCl buffer
Buffer 3	50 mM potassium phosphate, pH 7.2, 1 mM DTT, 0.5 M NaCl buffer
Buffer 4	50 mM potassium phosphate, pH 7.2, 1mM DTT buffer

2.3.5 SDS-polyacrylamide gel electrophoresis buffers

Running buffer (4 x stocks)

Tris	12.1 g
SDS	4.0 g
Glycine	57.6 g
H ₂ O to	1 litre

pH adjusted to 8.12 with HCl.

Separating gel buffer A (2 x stock)

Tris	90.6 g
SDS	2.0 g
H ₂ O to	1 litre

pH adjusted to 8.8 with HCl, and subsequently stored at 4°C.

Stacking gel buffer B (2 x stock)

Tris	30.24 g
SDS	2.0 g
H ₂ O to	1 litre
pH adjusted to 6.8 with HCl, and subsequently stored at 4 °C.	

Loading buffer (2 x stock)

Stacking gel buffer (2 x)	50 ml
SDS	4 g
Glycerol	20 ml
1 % Bromophenol blue	1 ml
2-mercaptoethanol	5 ml
H ₂ O to	1 litre

Ammonium persulphate (40 mg ml⁻¹)

Ammonium persulphate	0.4 g
H ₂ O to	10 ml
Prepared freshly on the day	

Separating gel (volumes for 23 ml)

Acrylamide (40 % including bis 19:1)	
10 %	5.6 ml
15 %	8.4 ml
Separating gel buffer (2 x)	11.4 ml
H ₂ O to	22.4 ml
The solution was de-aerated before the following were added	
40 mg ml ⁻¹ Ammonium persulphate	0.6 ml
TEMED	20 µl

Stacking gel (for 10 ml)

Stacking gel buffer (2 x)	5 ml
Acrylamide (40 % including bis 19:1)	1.3 ml
H ₂ O	3.6 ml
The solution was de-aerated before the following were added	
40 mg ml ⁻¹ Ammonium persulphate	0.25 ml
TEMED	24 µl

Coomassie Blue staining solution

Coomassie Brilliant Blue R250	0.4% (w/v)
Methanol	45% (v/v)
Glacial acetic acid	10% (v/v)
H ₂ O to	1 litre

De-staining solution

Methanol	10% (v/v)
Glacial acetic acid	14% (v/v)
H ₂ O to	1 litre

2.3.6 Western blotting buffers

Transfer buffer

25 mM Tris	6.06 g
192 mM Glycine	28.82 g
20% Methanol	400 ml
0.1% SDS (w/v)	2 g
H ₂ O to	2 litres

PBS

80 mM Na ₂ HPO ₄	57.5 g
20 mM NaH ₂ PO ₄	14.8 g
100 mM NaCl	5.84 g
H ₂ O to	5 litres

2.3.7 Lowry assay buffers

Solution A

CuSO ₄	0.05 g
Potassium tartrate	0.1 g
dH ₂ O to	100 ml

Solution B

Na ₂ CO ₃	2 g
NaOH	0.4 g
dH ₂ O to	80 ml

Genetic and microbiological techniques

2.4. Gene cloning

Gene cloning is a commonly used technique that typically consists of several steps: Amplification of DNA sequence of interests by the polymerase chain reaction. Insertion of the isolated gene in a vector. Transformation of the plasmid into host cells. Selection of the transformed cells.

2.4.1 Oligonucleotide primer design

The primers were designed by the Primer 3 software tool. The primers for PCR amplification were based on the sequence upstream and downstream of the desired open reading frame (ORF). For a fragment larger than 1000 bp, primers based on the coding sequence of the ORF were also needed for DNA sequencing. There are basic principles for primer design: 1) The primer length is normally between 20–25 bp for higher specificity and for the induction of restriction enzyme sites; 2) The GC contents are in the range of 40-60 % for optimum PCR efficiency; 3) Primers are not self-complementary or complementary to other primers in the reaction mixture; 4) The T_m of primers should be similar and aim in the region of 65°C and 70°C.

For amplifying the ORF sequence of AN2366.2, primers were designed as follows:

PCR primers:	5' - gct ata taa gga gtc aat tct - 3'
	5' – gct tgc ata tat tgg aac tgg - 3'
Sequencing primers:	5' – acc atg ata agg gcg gca - 3'
	5' – atg gct gcc agg gcg att - 3'
	5' – tgc cgc cct tat cat ggt - 3'
	5' – aat cgc cct ggc agc cat - 3'

2.4.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technique to amplify a parent DNA sequence across several orders of magnitude, generating thousands of copies of this identical DNA sequence (Mullis et al., 1994). The repeated thermal cycles consisted of DNA melting, primer and polymerase binding and DNA sequence elongation.

PCR reaction procedure for amplifying the ORF sequence of AN2366.2

Initiation stage	94°C for 4 minutes 55°C for 3 minutes 72°C for 2 minutes
Repeated cycles (35 cycles)	94°C for 3 minutes 55°C for 3 minutes 72°C for 2 minutes
Termination stage	94°C for 4 minutes 55°C for 3 minutes 72°C for 5 minutes 4°C for 4 hours

2.4.3 Agarose gel electrophoresis

All gels (0.8% (w/v) agarose) were poured into beds sealed at each end with autoclave tape and the wells formed by insertion of a Perspex comb. The Tris-acetate EDTA (TAE) agarose was melted in a microwave oven and then cooled down to 55°C before pouring. The gels were immersed in 1 x TAE buffer in an electrophoresis tank. The electrophoresis was carried out at 100 V for 45 mins at room temperature for efficient separation. The gel was then stained in 10 mg ml⁻¹ ethidium bromide for 15 mins and visualised under long wavelength ultraviolet (UV) light provided by a transilluminator. The image of the gel was generated using a Mitsubishi Video Copy Processor.

2.4.4 Purification of PCR products

The PCR products were prepared by modified Freeze Squeeze DNA isolation technique (Tautz and Renz, 1983). The gel bands of interest after agarose gel electrophoresis were excised and transferred into a 25 ml tube. The volume of 20 ml acetate solution was added into the tube for incubation at room temperature for 40 mins. The gel section was then transferred into a 0.5 ml tube previously punctured with a 21-gauge needle and plugged with siliconised glass wool, which was then inserted into a 1.5 ml tube before freezing at -80°C for minimum of 30 mins. The eluate from the 1.5ml tube was collected from each of 3 centrifugation steps of 12000 x g at room temperature for 10 mins in a bench top microfuge and pooled in a new 1.5 ml tube. A half volume of Phenol/chloroform/iso-amylalcohol (25:24:1) was added into the tube and mixed thoroughly and centrifuged again at 10,000 x g for 5 mins before carefully removing the top phase into a fresh tube. After the addition of 0.05 volumes of 5 M potassium acetate and 2.2 volumes of pre-cooled 95% ethanol (stored at -20°C), the mixture was stored at -20°C overnight. The supernatant was discarded after centrifugation at 10,000 x g for 30 mins and the pellet was washed with pre-cooled 70% ethanol (stored at -20°C) to remove any remaining salts. The pellet was dried down at 45°C for 30 mins and redissolved in dH₂O. The efficiency of the recovery of the DNA was checked by agarose gel electrophoresis.

2.4.5 Construction of plasmids

The construction of plasmid for DNA sequencing is based on the protocol of Agarose TOPO TA Cloning kit (Invitrogen). The TOPO cloning reaction mixture was described as following:

PCR product (500 ng)	2 μ l of 0.25 μ g μ l ⁻¹ stock
Salt solution (1.2 M NaCl, 0.06 M MgCl ₂)	1 μ l
dH ₂ O	2 μ l
TOPO vector	1 μ l

The mixture was incubated at room temperature for 10 mins before prior to its transformation into *E.coli* HB 101 cells. The plasmids were isolated by QIAGEN Plasmid Midi Kit from the transferred cells for following gene sequencing.

2.5 Transformation of *E.coli*

Chemically “one-shot” competent *E. coli* strains were purchased from biological companies (see section 2.29). The transformation procedure was based on the suggested protocols within the commercial transformation kits. The amounts of 2 μ l of plasmids were added into 10 μ l freshly thawed competent cells and 10 μ l pH 6.5 MOPS (morpholinopropanesulfonic acid) buffer plus 0.05 M CaCl₂. The mixture was placed on ice for 30 mins before “heat shock” at 42°C water bath for 30 secs. After 500 μ l Luria broth (LB) was added, the mixture was incubated at 37°C water bath for 1 h. The majority of the supernatant (400 μ l) from the centrifugation of 12,000 x g for 2 mins was removed and the resuspended solution was directly plated on Luria agar plates with selective antibiotics and grown at 37°C for single colonies.

2.6 Isolation of plasmid DNA from *E.coli*

The plasmid was transformed into chemically competent *E.coli* HB101 cells. After growing on selective Luria Broth agar plates at 37°C overnight, a scraping of colonies were used to inoculate 200 ml Luria Broth supplemented with the appropriate antibiotics. After 7 hours growth at 160rpm, 37°C, the cells were harvested by

centrifugation at 8000 x g for 10 mins. Plasmid DNA was isolated from the cell pellet by QIAGEN Plasmid Midi Kit.

2.7 Bulk growth of *E.coli* cultures growth in liquid media

Single colonies were picked individually by sterile toothpicks and used to inoculate 10 ml LB media with selective antibiotics and grown at a desired temperature overnight without shaking. The 10 ml culture were then inoculated into 500 ml LB media containing antibiotics and grown at 160rpm, 25°C or 30°C up to 15 hours until an attenuation of approximately 0.6-0.8. At this point recombinant protein production was induced by the addition of an appropriate inducer and allowed to proceed for 5-15 hours depending on the growth conditions. After the induction efficiency had been checked by SDS-PAGE electrophoresis of boiled cells, cell pellets were harvested by repeated centrifugations of 12,000 x g, 6 mins at 4°C and stored at -20°C.

2.8 Maintenance of *A. nidulans* strains

Working stocks of strains of *A. nidulans* were maintained on AMM plates supplemented with nitrogen source, carbon source and appropriate vitamins and stored at 4°C. Before use, the genotypes of the strains were checked by appropriate growth tests.

2.9 Preparation of conidial spore suspensions

A sterilised loop was immersed in Tween saline (0.01% (v/v)) and then used to collect conidia from plates of *A. nidulans* by gently sliding the loop across the surface of the mycelia. After suspending in 100 µl Tween saline, conidia were spread on the surface of fresh plates using a sterile glass spreader and incubated at 37°C for approximately 72

hours. Conidia were then harvested by flooding the surface of the plate with 10 ml Tween saline and rubbing gently with a sterilised disposable Pasteur pipette. After centrifuged at 8000 x g for 5 mins, the supernatant was removed and the conidia were resuspended into fresh Tween saline and stored at 4°C for 6-8 weeks. The attenuation at 550 nm of a 10-fold dilution of the conidial suspensions was measured and used to estimate the spore concentration from a standard graph generated from spore suspension of known concentration.

2.10 Mycelial growth in liquid media

The amounts of 2×10^8 spores were grown in 200 ml AMM liquid media in 2 L long baffled flasks with 10 mM ammonium/nitrate, 0.4% (w/v) carbon source as well as other necessary supplements at 180rpm, 37°C for 18-19 hours to generate healthy mycelia. The mycelia were then transferred into new media with no nitrogen source for 4 hours starvation to induce protease production prior to harvest. Using the same growth conditions, spores were grown with 2.5 mM, 5 mM, 10 mM, 20 mM or 40 mM ammonium/nitrate to investigate the effect of nitrogen source concentration on the production of proteases. Spores were also grown with 40 mM ammonium under the nitrogen sufficient condition to guarantee the absences of proteases and then starved of nitrogen for different times by subsequent incubation in nitrogen free media. To test the effect of carbon starvation on protease production, the concentration of glucose for growth was reduced from 0.4% (w/v) to 0.1% (w/v). Harvested mycelia were dried by absorbing extra liquid with tissue and wrapped in foil, flash frozen with liquid nitrogen before storage at -80°C.

Biochemical techniques

Edman sequencing, MALDI-TOF Mass spectrometry and whole protein LC-MS were performed in Pinnacle lab, Institute of Cell and Molecular Biosciences, Newcastle University, United Kingdom.

2.11 SDS-polyacrylamide gel electrophoresis

Protein samples mixed with an equal volume of loading buffer were denatured at 95°C for 6 mins. Samples were electrophoresed at 35 V per gel for approximately 30 mins until the loading dye at the bottom of the gel. Proteins were made visualised by staining with Coomassie Blue R250.

2.12 Testing the solubility of overproduced recombinant proteins

To determine if the overproduced recombinant proteins were soluble, cell pellets from 1 ml of induced *E. coli* cultures were resuspended in 1 ml of each buffer (see section 2.3.4). The cell suspensions were disrupted by sequential bursts (6 x 15 secs) of sonication (amplitude 5 μ) in an ice/ water mix. After centrifugation of 10,000 x g for 10 mins at room temperature, the soluble fractions were transferred to fresh tubes and the insoluble materials was resuspended in 500 μ l dH₂O. Both soluble and insoluble fractions were analysed by SDS-PAGE electrophoresis to facilitate the determination of the recombinant protein solubility.

2.13 Preparation of cell free extracts of *A.nidulans*

Cell free extracts were obtained from 0.5 g of frozen mycelium (more mycelia were required for protease purification as stated out in following chapters), by grinding to a fine powder in a mortar pre-cooled with liquid nitrogen. The powder was then resuspended in 2 ml extraction buffer (0.1 M Tris-HCl pH 7.5, 1 mM DTT), and gently shaken on ice for a minimum of 60 mins. Cellular debris was removed by centrifugation at 10,000 x g for 10 mins \times 2 at 4°C in 1.5 ml centrifuge tubes, the supernatant was carefully transferred to fresh tubes and flash frozen in liquid nitrogen, and stored at -80°C until required for subsequent assays.

Large amounts of cell free extracts were obtained modifying the same procedure. In this case up to 100 g of ground mycelia were stirred on ice for 60 mins in volumes up to 500 ml extraction buffer. The soluble fraction was recovered by filtering the stirred mycelial suspension through muslin cloth and subsequently centrifuging the filtered suspension at 10,000 x g for 42 mins at 4°C.

2.14 Determination of protein concentration (Lowry Assay)

Protein concentrations were determined using the method of Lowry (Lowry et al., 1951). The volume of 1 ml of solution mixture (1 A: 4 B) was added per protein sample to be quantified. Typically, samples were measured in duplicate with 5 μ l and 10 μ l of each diluted to 200 μ l with de-ionised water in order to confirm dose dependency. A series of BSA standards (0-200 μ g ml⁻¹) were used to produce a standard curve. After 15 mins incubation at room temperature, 100 μ l of freshly diluted acid solution of Folin

Ciocalteu's phenol reagent (adding 1.3 volumes of 1 M HCl) was added and the samples were incubated at room temperature for 30 mins. Protein concentration was determined at 495 nm against a protein free blank.

2.15 Proteolysis assay

This assay was used to detect the presence of proteases in cell free *A. nidulans* extracts using recombinant NmrA as the substrate. After the protein concentration been determined by Lowry assay, 1 μg cell free extracts were incubated with 2 μg NmrA protein ($0.4 \mu\text{g} \mu\text{l}^{-1}$) at a final volume of 10 μl in 50 mM potassium phosphate, pH 7.2, 1 mM DTT buffer at 25°C for 30 mins and the proteolysis products were analysed by SDS-PAGE using 10% or 15% separating gels. To analyse chromatographically separated protease activities, 10 μl fractions were incubated with 2 μg NmrA ($0.4 \mu\text{g} \mu\text{l}^{-1}$) at 25°C for 30 mins at a final volume of 15 μl . Extended incubation up to 6 hours was necessary to investigate the inherent resistance of NmrA to extensive proteolysis.

2.16 Casein Assay for proteolysis

This procedure was based on protocol of E6638 *EnzCheck* Protease Assay kit (Invitrogen). All the reagents were stored at -20°C and prepared on ice for the assay while the measurements were carried at room temperature. The stock solution of the BODIPY FL casein was prepared at the concentration of 1.0 mg ml^{-1} by adding 0.2 ml PBS directly to the vial containing 0.2 mg lyophilised substrate and stored in the dark. To check for dose dependency, 5 μg and 10 μg of each sample were tested individually with 10 μl stock solution of casein, at a final volume of 1 ml with 50 mM potassium phosphate, pH 7.2, 1 mM DTT buffer for dose dependency. After thorough mixing,

the change in fluorescence was read by Varian Eclipse Fluorescence Spectrophotometer. One unit of protease activity was defined as a fluorescence change of 100 units per minute. The programme was set up as 505/513 nm of excitation/emission maxima and collected readings every minute from 0 to 10 mins. Reactions were considered to be dose-dependent if the error in the rates of change or the two concentrations of protease tested was less than 15%.

2.17 Protein purification

The protein purification was performed by a combination of low pressure chromatography and FPLC. The basic procedure is to flow solution containing proteins on different chromatographic columns. Different proteins interact with different column matrix or have different binding affinity and thus can be separated by the different time of passing through the column or eluted from the column by different conditions.

2.17.1 Isolation of proteases PNM1A and PNM1B

The amounts of 25 g of frozen mycelia grown at 37°C in minimal media containing 10 mM ammonium and subsequently transferred to nitrogen free media for 4 hours, was ground to a fine powder in liquid nitrogen for cell free extraction. The cell free extracts were filtered through a 0.45 µm Whatman Filter and chromatographed on a 5 ml HiTrap DEAE FF column in conjunction with an AKTA Explorer chromatography station (GE Healthcare). A 20 column volume 0.0 -1.0 M NaCl gradient in Buffer 1 was used to elute bound proteins in 2 ml fractions after a 20 column volume wash. The column flow through and wash was collected as 50 ml fractions. Protease-containing fractions were identified by proteolysis assay with NmrA protein

and SDS-PAGE electrophoresis. Appropriate pools containing the proteases PNM1A and PNM1B were air-pressured or using vivaspin concentrator to concentrate 50-fold and further chromatographed on a Superdex 200 HR 10/30 column (GE Healthcare) eluted in Buffer 2 and collected 0.5 ml fractions. After identification by proteolysis assay and SDS-PAGE electrophoresis, fractions containing PNM1A and PNM1B were pooled individually and stored at -20°C for further experiments.

Trichloroacetic acid (TCA) precipitation was used occasionally for further protein concentration when the volumes of the protein solutions were too small for air-pressure concentration or vivaspin concentration: After 0.25 volumes of 100% (w/v) TCA had been added, the protein solution was incubated at 4°C for 10 mins. The protein pellets from the subsequent centrifugation of 10,000 x g for 10 mins were then washed with fresh acetone repeatedly for 3 times. The final pellets were air-dried and then dissolved into protein buffers for further experiments.

2.17.2 Purification of AreA Zf fragment

E. coli strain BL21 AI containing plasmid pMUT146 was grown in LB media containing 100 µg mg⁻¹ ampicillin at 30°C until an attenuation at 550 nm of 0.8. At this point the cultures were made up to 0.2% (w/v) arabinose and incubated for a further 5 hours at which point the cells were harvested by centrifugation at 10,000 x g for 10 mins. The amounts of 25 g of cells were resuspended in 500 ml Buffer 4 containing 1 mM benzamidine. After sonication (amplitude 15 µ) for 20 mins, the cell free extracts were prepared by centrifugation of 10,000 x g for 42 mins at 4°C. An immobilised metal chelating Sepharose column that had been charged with zinc binding capability was equilibrated with Buffer 4 for soluble proteins binding. The column was washed

with 500 ml Buffer 4, followed by elution with a 0.0-3.0 M imidazole gradient and 5 ml fractions collection. The AreA-containing fractions were identified by SDS-PAGE and pooled appropriately. This pool of proteins contained some precipitate which was removed by centrifugation of 10,000 x g for 15 mins at 4°C. The supernatant was subsequently dialysed overnight against 3 x 5 L Buffer 4, filtered through a 0.45 µm filter and stored at -80°C.

2.17.3 Purification of NmrA protein

The NmrA protein was prepared as described previously (Nichols et al., 2001). *E. coli* strain BL21 DE3 containing plasmid pTR121 was grown in LB media with addition of 0.1 mg mg⁻¹ ampicillin at 30°C until an attenuance at 550 nm of 0.8, following a 5-hour induction with 0.2 mg ml⁻¹ IPTG before harvest. The amounts of 25 g of cells were resuspended in 500 ml Buffer 4 containing 1 mM benzamidine. After sonication (amplitude 15 µ) for 20 mins, the cell free extracts were prepared by centrifugation of 10,000 x g for 42 mins at 4°C. The soluble proteins were separated by the DEAE Sephacel column with elution of 0.0-1.0 M NaCl gradient in Buffer 4 by collecting 10 ml fractions. NmrA-containing fractions were identified by SDS-PAGE and pooled for subsequent ammonium sulphate (AmSO₄) fractionation. AmSO₄ was slowly added to the collected pool until the final concentration was 50% (w/v) saturation and, after thoroughly stirring on ice for further 30 mins, the pellets were harvested by centrifugation of 10,000 x g, for 42 mins at 4°C. The pellets were then redissolved in 200 ml Buffer 4 containing 1 M AmSO₄ and applied to a phenyl Sepharose column equilibrated in Buffer 4 containing 1 M AmSO₄. Following a 500 ml wash with Buffer 4 containing 1 M AmSO₄ the proteins were eluted by 1 L Buffer 4 with a linear

gradient of 1.0-0.0 M AmSO_4 collecting 10 ml fractions. Appropriate fractions identified by SDS-PAGE were pooled appropriately and dialysed overnight against 3 x 5 L Buffer 4. The dialysed protein was then applied to a hydroxyapatite column equilibrated in Buffer 4. Following a wash with 500 ml of Buffer 4 the column flow through was analysed by SDS-PAGE and appropriate NmrA-containing fractions were pooled, filtered through a 0.45 μM filter and stored at -80°C .

2.17.4 Purification of NM1B+C term of NmrA protein

The proteolytic NM1B+C term was generated by digesting full length NmrA with purified PNM1B in the presence of 0.5 mM NAD^+ in Buffer 4 at 25°C . Approximately 100 mg of recombinant NmrA was digested with 4 ml purified PNM1B (active pool collected from fractions after Superdex column) at 25°C for 1-2 hours in the presence of 0.5 mM NAD^+ . The extent of proteolysis was monitored by SDS-PAGE and when no less than 95% NmrA had been converted into NM1B, further digestion was inhibited by the addition of benzamidine to final concentration of 10 mM. The proteolysis products were then loaded on to MONO Q 10.100 GL FPLC column. Subsequently the MONO Q column was washed with 3 column volumes of Buffer 4 to remove the benzamidine, and the digested NmrA was separated from the PNM1B by elution with a 20 column volume 0.0-1.0 M NaCl gradient at the rate of 3 ml min^{-1} and collected 2 ml fractions. After suitable fractions had been pooled, NaCl was removed by dialysis against Buffer 4 and typically yielded approximately 25 mg of the proteolytic fragment of NM1B+C. Proteins were filtered through a 0.45 μM filter and stored at 4°C for further experiments.

2.18 Protein measurements

Measurements of the optical densities of protein were made using a Shimadzu 1601 double beam UV visible range spectrophotometer. These values were expressed in terms of absorbance at wavelength of 280 nm determined from a scan of 240-400 nm. Protein concentrations were subsequently determined from their theoretical calculated molecular absorption coefficients calculated by the Vector NTI Suite of programmes (Informax, Inc.).

2.19 Urea gradient protein refolding

In order to solubilise the insoluble recombinant proteins, 6.0 M urea was added to the sonication buffer (50 mM potassium phosphate pH7.2, 1 mM DTT). This resulted in unfolding of the recombinant protein and refolding was attempted by the slow removal of the urea by dialysis.

The amounts of 50 g cells overproducing the recombinant protein of interest were thoroughly sonicated (amplitude 15 μ) in 1L Buffer 4 for 20 mins. After the centrifugation of 10,000 x g for 42 mins at 4°C the supernatant was discarded. The pellet was washed twice in Buffer 4 and then resuspended into 200 ml Buffer 4 with 6 M urea. With high concentration of urea, the insoluble protein is unfolded thus transferred into soluble portion. Following a second centrifugation step at 10,000 x g for 42 mins at 4°C, the unfolded protein containing supernatant was dialysed into 5 L of Buffer 4 in which the concentration of urea was reduced from 6.0-0.0 M in 0.5 M reduction per step. Each dialysis step took at least 12 hours. After the final dialysis

step, the solution was passed through a 0.45 μm filter and increasing loadings of the soluble protein analysed by SDS-PAGE using a 10% separating gel.

2.20 Western blotting

The method of western blotting involves the transfer of proteins from the SDS-PAGE gel to nitrocellulose, which can then be probed with antibodies specific to particular amino acid sequences (Burnette, 1981).

Following SDS-PAGE, the separating gel was equilibrated by immersion in 2 x 100 ml pre-chilled transfer buffer at 4°C for 30 mins. Two sheets of Bio-RAD extra thick blotting paper were placed in transfer buffer for 15 mins. One filter pad was placed on the anode plate of the Geneflow transblotter semi-dry filter cell. A wet filter paper, the nitrocellulose membrane, SDS-PAGE gel and a second piece of wet filter paper were sequentially placed to form a sandwich. Air bubbles were excluded by rolling a glass rod across the sandwich, and then the cathode plate was then placed on top. The proteins were blotted onto the nitrocellulose by running 15 V through the sandwich for 70 mins.

After blotting, the nitrocellulose membrane was placed in 20 ml blocking buffer (PBS + 0.1% (v/v) Tween 20 + 10% (w/v) skimmed milk) and incubated for 2 hours at room temperature on an orbital shaker or 16 hours standing still at 4°C. This resulted in all non-specific binding sites of the antibody being blocked with irrelevant proteins. The nitrocellulose membrane was then washed 3 times with 20 ml wash buffer (PBS + 0.5% (v/v) Tween 20) to remove residual traces of the blocking buffer.

The western blot was probed in 2 stages: first by incubating the membrane with an unlabelled primary antibody specific to the target protein, for 1 hour at room temperature on an orbital shaker. The primary antibody was diluted in 10 ml PBS + 0.1% (v/v) Tween 20 + 5% (w/v) skimmed milk. Non-specifically bound antibody was then washed off by 100 ml wash buffer for 15 mins for three times at room temperature. Then, the HRP labelled secondary antibody was diluted in 8 ml PBS + 0.1% (v/v) Tween 20 + 5% (w/v) skimmed milk, and incubated with the membrane for 1 hour at room temperature on an orbital shaker. The membrane was then washed three times with 100 ml wash buffer for 15 mins at room temperature.

The antigen-antibody-antibody complexes on the nitrocellulose membrane were then detected by ECL Western blotting detection analysis system (GE Healthcare). After the extra solution was drained from the membrane by tissue, it was placed on Saran Wrap with the protein side up. Equal volumes of detection solution 1 and 2 were used for a final volume of 4 ml mixture required to cover the membrane. After incubation for 1 min at room temperature, the extra detection reagent was removed from the membrane by tissue. The membrane was then wrapped between two sheets of cellulose acetate and the air bubbles smoothed out. The wrapped blot was then placed protein side up in an X-ray film cassette. The blot was developed after 3 mins exposure time.

2.21 Antibodies

The anti-NmrA antibody and anti-Type II-dehydroquinases antibody, gifts from Professor Hawkins, was raised from rabbits under standard protocols (Harlow and Lane, 1988) and purified and affinity tested in advance (Johnson, 2008). The anti-NmrA

antibody was used at 1:1600 dilutions and the HRP labelled secondary antibody was used at 1:2000 dilutions. The anti-Type II-dehydroquinases antibody was used at 1:6400 dilutions and the HRP labelled secondary antibody was used at 1:1000 dilutions.

2.22 Edman sequencing

Proteins separated by SDS-PAGE gels were electroblotted (150 mA for 60 mins) onto PVDF membranes with 10 mM CAPS, pH 11.0 buffer (cyclohexylamino-1-propanesulphonic acid) containing 10% (v/v) methanol. Membranes were then stained with Coomassie Blue R250 and the bands were excised for N-terminal Edman protein sequencing using an LF3000 sequencer (Beckman). Automated Edman sequencing was carried by Joe Gray in the Pinnacle proteomics facility.

2.23 Protein extraction for mass spectrometry

De-stained gel bands were diced into small pieces (about 1 mm²) and placed in a 0.2 ml tube. A solution consisting of 50% (v/v) hexafluoro-2-propanol (HFIP), 20% (v/v) formic acid (aq) was added to cover the gel slices (around 50 µl). Extraction was performed for 2 hours at room temperature before the supernatant was removed to a clean 0.2 ml tube and reduced to 10 µl *in vacuo* before being subjected to LC/ MS or MALDI analysis. These procedures were carried out by Joe Gray in the Pinnacle proteomics facility.

2.24 MALDI-TOF Mass spectrometry

MALDI-TOF analysis was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (Biosystems). The instrument was operated in linear mode for whole protein analysis and in reflectron mode for peptide digests. Monoisotopic peptide

mass fingerprints were generated and average protein masses (MW) assigned using Data Explorer software (Biosystems). For whole protein mass measurements four sequential 0.5 μl aliquots of intact protein extract were dried onto a target plate pre-spotted with 1 μl of sinapinic acid matrix solution (10 mg ml^{-1} in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid (2 μl). The tryptic digest data was analysed by two independent means, Mascot identity database searching and BLAST homology searching of *de novo* generated sequences in conjunction with the program DeNovoX. The *de novo* sequencing of peptides was attempted from their MS/MS spectra without prior reference to any database. The output of this process was manually inspected and the best 25-30 peptide MS/MS sequences were extracted to a list for BLAST homology searching in both directions using the Swiss-Prot database. These procedures were carried out by Joe Gray in the Pinnacle proteomics facility.

2.25 Whole protein LC/MS

The 10 μl protein extract was diluted to 50 μl using 0.1% (v/v) formic acid (aq) and the acidified protein solution loaded onto a home-made trap column (approximate dimensions: 2 mm long x 1 mm diameter) packed with C18 sorbent (3 M Empore, 60 Å pore, 12 μm particle size). Loading was performed using the syringe pump of the mass spectrometer at 10 $\mu\text{l min}^{-1}$. The loaded trap column was then flushed with 100 μl 0.1% (v/v) formic acid to remove non-bound material. For electrospray protein analysis the trap was switched in-line with the flow of an HPLC (Agilent 1100) pumping at 100 $\mu\text{l min}^{-1}$ and flow-split to produce a trap column flow of 10 $\mu\text{l min}^{-1}$. Buffer A contained 0.1% (v/v) formic acid (aq) and buffer B was acetonitrile with 0.1% (v/v) formic acid. A linear elution gradient from 4% to 64% of buffer B in 22 mins was used to elute the bound protein from the C-18 trap. The flow from the column

was coupled to an “Ion Max” ion source (ThermoElectron) and sprayed directly into an LTQ-FT mass spectrometer (ThermoElectron). LC/MS data was collected using the FTMS analyser in full scan and normal mass range mode at a resolution of 200,000 (at $m/z = 400$). The mass spectrometer collected data from scans over the mass range $m/z = 300-2000$. Mass spectrum plots and spectral deconvolution were generated using the QualBrowser program (ThermoElectron). Protein spectra were deconvoluted to determine the average molecular mass of the protein assuming “averagine” (0.2678% sulphur) composition. These procedures were carried out by Joe Gray in the Pinnacle proteomics facility.

Biophysical techniques

2.26 Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry is a non-destructive and non-invasive (label-free) technique that can be used to give a direct measure of the K_D and additionally provides information on the enthalpy (ΔH) and stoichiometry (n) of ligand binding (Freire et al., 1990). In these ITC experiments one protein solution is placed in a reaction vessel and a second protein is titrated in from a syringe whilst constantly stirring the mixture. Any heat absorbed (endothermic reaction) or released (exothermic reaction) when ligand binds to the protein is measured directly. Standard Gibbs free energy (ΔG_0) and entropy (ΔS_0) of ligand binding are subsequently calculated from the following equations:

$$\Delta G_0 = -RT \cdot \ln K_b = +RT \cdot \ln K_D = \Delta H - T \cdot \Delta S_0$$

ITC experiments were performed at 25°C using a high precision VP-ITC system (Microcal). Proteins were dialysed into 50 mM potassium phosphate pH 7.2, 1 mM DTT, and the concentrations of the reagents used in the injector and cell are shown in the legends of Fig 6.9 and Fig 6.10. The heat evolved following each 10 µl injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction was obtained as the difference between the heat of reaction and the corresponding heat of dilution. Analysis of data was performed by Microcal Origin software.

2.27 Circular dichroism (CD) spectroscopy

The Circular dichroism (CD) is the difference in the absorption of left-handed circularly polarised light (L-CPL) and right-handed circularly polarised light (R-CPL).

Circular dichroism = $\Delta A(\lambda) = A(\lambda)_{\text{LCPL}} - A(\lambda)_{\text{RCPL}}$, where λ is the wavelength

The Circular dichroism spectroscopy is used to measure the CD of molecules over a range of wavelengths, commonly for the investigation of the secondary structure of proteins (Rodger and Norden, 1997).

The far-UV CD spectrum of NM1B+C term was recorded from an average of 5 accumulative scans at room temperature from 250 nm to 185 nm using a Jasco J-810 spectropolarimeter and cuvettes with a 0.01 mm path length. Samples of NM1B+C term were used at concentration of approximately 15 µM in 50 mM potassium phosphate pH 7.2, 1 mM DTT.

2.28 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is a technique for direct measurement of heat changes in biomolecules during temperature increase or decrease (Watson et al., 1964). The protein molecules in solution are in equilibrium between the native (folded) conformation and its denatured (unfolded) state. The DSC measures the enthalpy (ΔH) of protein unfolding due to heat denaturation. The higher the thermal transition midpoint, when 50% of the protein molecules are unfolded, the more stable the molecules. The change in heat capacity (ΔC_p) for denaturation is primarily due to changes in hydration of side chains that were buried in the native state, but become solvent exposed in the denatured state.

The DSC experiments were performed using Microcal VP-DSC instruments from 25°C to 70°C at the rate of 90°C per hour. Proteins were dialysed into 50 mM potassium phosphate pH 7.2, 1 mM DTT buffer at a concentration of 20 μ M. The DSC experiments were repeated with addition of 3 mM NAD^+ under the same identical conditions. Analysis was performed using the non-two-state model with the Microcal Origin software.

2.29 Strain and plasmids used

Strains used in this work

<i>E. coli</i> strain	Genotype	Source or reference
HB101	F-, <i>thi</i> -1, <i>hsdS</i> ₂₀ (<i>r</i> _B ⁻ <i>m</i> _B ⁻), <i>supE</i> 44, <i>recA</i> 13, <i>ara</i> -14, <i>leuB</i> 6, <i>proA</i> 2, <i>lacY</i> 1, <i>galK</i> 2, <i>rpsL</i> 20 (<i>str</i> ^R), <i>xyl</i> -5, <i>mtl</i> -1	Promega
BL21-AI	F-, <i>ompT</i> , <i>hsdS</i> _B (<i>r</i> _B ⁻ <i>m</i> _B ⁻), <i>gal</i> dcn, <i>areB</i> :: T7RNAP-tetA	Invitrogen
BL21 (DE3)	F-, <i>ompT</i> , <i>hsdS</i> _B (<i>r</i> _B ⁻ <i>m</i> _B ⁻), <i>gal</i> dcn (DE3)	Novagen
BL21-Codon⁺-RIL	<i>E. coli</i> B F-, <i>ompT</i> , <i>hsdS</i> (<i>r</i> _B ⁻ <i>m</i> _B ⁻), <i>dcm</i> ⁺ , Tetr, <i>gal</i> , λ(DE3), <i>endA</i> , Hte[<i>argU proL Camr</i>],[<i>areU ileY leuW Strep/Spectr</i>]	Stratagene
BL21 (DE3) pLysS	F-, <i>ompT</i> , <i>hsdS</i> _B (<i>r</i> _B ⁻ <i>m</i> _B ⁻), <i>dcm</i> , <i>gal</i> ,λ(DE3), pLysS, Cm ^r	Promega
Origami 2	Δ(<i>ara-leu</i>)7697,Δ <i>lacX</i> 74,Δ <i>phoA</i> , Pvu II, <i>phoR</i> , <i>araD</i> 139, <i>ahpC</i> , <i>ale</i> , <i>galK</i> , <i>rpsL</i> , F'[<i>lac</i> ⁺ <i>lacI</i> ^q <i>pro</i>], <i>gor522</i> :: <i>Tn</i> 10, <i>trxB</i> (<i>Str</i> ^R , Tet ^R)	Novagen
Origami 2 (DE3)	Δ(<i>ara-leu</i>)7697,Δ <i>lacX</i> 74,Δ <i>phoA</i> , Pvu II, <i>phoR</i> , <i>araD</i> 139, <i>ahpC</i> , <i>ale</i> , <i>galK</i> , <i>rpsL</i> , F'[<i>lac</i> ⁺ <i>lacI</i> ^q <i>pro</i>] (DE3), <i>gor522</i> :: <i>Tn</i> 10, <i>trxB</i> (<i>Str</i> ^R , Tet ^R)	Novagen
TOP10	F- <i>mcrA</i> , Δ(<i>mrr-hsdRMS-mcrBC</i>), Φ80 <i>lacZ</i> Δ <i>M</i> 15, Δ <i>lacX</i> 74, <i>recA</i> 1, <i>araD</i> 139, Δ(<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (<i>Str</i> ^R), <i>endA</i> 1, <i>nupG</i>	Invitrogen

<i>A. nidulans</i> strain	Genotype	Source of reference
R153	<i>wa3 pyroA</i> 4	(Roberts, 1963)
R21	<i>ya2 pabaA</i> 1	(Roberts, 1963)
NKRB3-2	Δ <i>areA ya2 pyroA</i> 4 <i>riboB</i> 2:: <i>ribo</i> ⁺	Provided by Davis M.
MH8935	Δ <i>nmrA ya2 pyroA</i> 4 <i>bleoR</i> <i>lacZ amdS</i> ⁻	Provided by Davis M.
JK2050	<i>bio</i> ⁻ <i>ribo</i> ⁻	Provided by Kinghorn J.
AJC100	<i>paba</i> ⁻	Provided by Kinghorn J.

Plasmids used in this work

Plasmid	Description	Source or reference
pILJ16	pUC8 derivative carrying the <i>A. nidulans argB</i> gene	(Johnston et al., 1985)
pTR121	Recombinant pTrc 99a containing a cDNA copy of the entire protein-coding region of the <i>nmrA</i> gene from <i>A. nidulans</i> strain R 153.	(Cocklin, 2001)
pMUT146	pET3 with the coding region of <i>areA</i> for residues 662-727 followed by 6 His tag inserted	Blue Heron
pNUFC344	pUC18 carrying the <i>A. nidulans argB</i> gene and <i>nmrA</i> gene constructed between the <i>qutE</i> promoter and terminator	(Dodds, 2000)
pMUT242	pET3 with the coding region of <i>nmrA</i> for residues 1-283 inserted	Blue Heron
pMUT248	pET3 with the coding region of AN2366.2 for residues 1-249 inserted	Blue Heron
pMUT249	pET3 with the coding region of AN2366.2 for residues 20-249 inserted	Blue Heron
pCR4-TOPO	Plasmid vector has a single 3' thymidint (T) for PCR products cloning	Invitrogen

Chapter 3 NmrA overproduction is correlated with an inhibited growth phenotype

3.1 Introduction

As described in Chapter 1, the signal transduction pathway mediating nitrogen metabolite repression is complex and operates through a range of mechanisms that include the control of transcription and post-translational modulation. For example, the production of *areA*-specific mRNA is partly under the control of the AreA protein and the stability of the *areA*-specific message is dependent on the nitrogen status of the growth medium. The 12 C-terminal residues of AreA protein are essential for its transcriptional activation function, and the stability of *areA*-specific mRNA is mediated through its 3'-untranslated region. The half-life of *areA*-mRNA is approximately six times longer in mycelium grown under nitrogen derepressing conditions (that is utilising the secondary nitrogen sources such as nitrate) than repressing conditions (that is utilising preferred nitrogen source ammonium) (Platt et al., 1996). AreA was the first GATA factor discovered to be directly involved in chromatin remodelling, and its binding to the essential GATA site 5' of linked *niiA* and *niaD* genes is responsible for drastic nucleosomal rearrangements (Muro-Pastor et al., 1999).

The transcription activating function of AreA is post-translationally modulated through an interaction with the repressor protein NmrA. However, the molecular mechanism whereby NmrA exerts its repressing function on AreA was unclear at the time this research started. *In vitro* and *in vivo* work showed that the NmrA homologue in *N.*

crassa, Nmr1, specifically bound to two distinct regions of the AreA homologue Nit2 to prevent the binding of Nit2 to DNA. Full length Nmr1 protein was fused to the GAL4 DNA-binding domain and the entire Nit2 protein was fused to the GAL4 activation domain (Xiao et al., 1995). Expression of these constructs in yeast activated β -galactosidase production indicating that Nmr1 and Nit2 were interacting *in vivo*. These experiments were extended by screening for interactions between Nmr1 and fragments of Nit2. Only fragments of Nit2 beginning at the zinc finger and extending to the C-terminus showed a strong positive result in this assay, however, the data implied that the extreme C-terminal region of Nit2 (lacking the zinc finger region) could interact with Nmr1. These results were subsequently checked by *in vitro* experiments in which various segments of Nit2 were tested for interaction with fragments of Nmr1. In this analysis the GST- or His 6-fusions of the fragments of Nmr1 and Nit2 were used in conjunction with glutathione-agarose to screen for protein-protein interactions. The results of these experiments were consistent with the yeast two-hybrid experiments and confirmed that the zinc finger region of Nit2 was involved in the interaction with Nmr1. However, the interaction between Nmr1 and the extreme C-terminal region of Nit2 was not detected in these experiments. N-terminally deleted Nmr1-GST fusions and fragments of Nit2 containing the zinc finger region were purified and used in combination in electrophoretic mobility shift assays (EMSA). In these EMSA the fragments of Nmr1 appeared to prevent the binding of the Nit2 fragments to GATA-containing DNA. These results contrasted with results obtained with *in vitro* experiments using the NmrA and AreA proteins from *A. nidulans*. In these experiments full length non-fusion NmrA was used in conjunction with an N-terminally His 6-tagged fragment of AreA that started at the zinc finger region and extended to the C-terminus. The interaction between these proteins and a GATA-containing double

stranded oligonucleotide were analysed by Isothermal titration calorimetry (ITC) and fluorescence polarisation (FP) experiments. These ITC and FP experiments showed that the zinc finger-containing C-terminal fragment of AreA bound to full length NmrA with a 1:1 stoichiometry, thereby implying a single binding site. Furthermore, the C-terminal AreA fragment was able to bind to the GATA-containing motif in the presence of an excess of NmrA (Lamb et al., 2004). These results indicate that NmrA may not exert its repressing function by directly preventing AreA binding to DNA, but in an indirect way, such as regulating AreA's entry into the nucleus.

Given these contrasting conclusions from the two sets of experiments, the effect of overproducing the NmrA protein *in vivo* was investigated. The transcription stimulating function of AreA is predicted to be inhibited by overproduction of NmrA *in vivo* thereby causing a phenotype similar to that of an *areA* deletion strain. This is because elevated levels of NmrA are predicted to trap the pool of AreA in the transcriptionally inactive binary complex.

3.2 Growth phenotype of wild type and NmrA over-producing transformants in the presence of the preferred nitrogen source ammonium

In order to test the hypothesis that NmrA binding to AreA inhibits AreA activation, NmrA over-producing transformants were constructed. The wild type *nmrA* gene was placed under the control of the quinate-inducible *qutE* promoter and terminator and subcloned into a plasmid harbouring the *argB* gene, the final plasmid was designated pNUFC344 and the details of its construction have been described previously (Johnson, 2008). Two NmrA wild type strains AJC100 and JK2050 were chosen for transformation to check for any effects of differences between genetic backgrounds. Empty plasmid pILJ16, which lacks the *nmrA* gene but carries the *argB* gene was used to generate control transformants. The advantage of producing transformants using pNUFC344 is that it allows over-expression of the heterologous *nmrA* in a controlled manner that is independent of the signals mediating nitrogen metabolite repression. The disadvantage of using this plasmid is that transformants are generated by random integration into the host genome; raising the possibility that integration into genes controlling nitrogen metabolite repression may occur. However, as transcription of the heterologous *nmrA* gene can be effectively switched off by growing on the catabolite repressing carbon source glucose, suitable transformants can be identified by their wild-type nitrogen metabolite repression growth response when growing on secondary nitrogen sources in the presence of glucose.

Transformants were selected for their ability to grow on AMM plates lacking arginine and using glucose as carbon source and ammonium as nitrogen source. After purification from single spores, the growth phenotypes of transformants on AMM

supplemented with glucose and the secondary nitrogen source nitrate were determined. This work was previously carried by Christopher Johnson as part of his PhD thesis (Johnson, 2008). From all the transformants tested, ten that showed a wild-type phenotype when growing on primary or secondary nitrogen sources with glucose as the carbon source were chosen for further analysis.

The ten transformants, two controls from both strains (i.e. transformed with pILJ16), a *nmrA* deletion strain and an *areA* deletion strain (NKRB3-2) were tested for their growth phenotypes on AMM supplemented with different carbon source combinations. The carbon source combinations used were glucose (strongly catabolite repressing; transcription from the *qutE* minimal in the absence of quinate), glucose and quinate (strongly catabolite repressing, transcription from the *qutE* promoter maximal), glycerol (less strongly catabolite repressing; transcription from the *qutE* promoter minimal in the absence of quinate), glycerol and quinate (less strongly catabolite repressing, transcription from the *qutE* promoter maximal) and quinate (transcription from the *qutE* promoter maximal).

Spores were grown at 37°C for 48 hours before scoring the phenotypes. When ammonium was used as nitrogen source, all transformants had a phenotype similar to the controls whether using glycerol, glucose or quinate or two in combination as carbon source. Typical results are shown in Fig 3. 1. These observations demonstrate that these transformants are suitable for further analysis of their growth phenotypes on secondary nitrogen sources in the presence of quinate and how this correlates with the relative level NmrA overproduction.

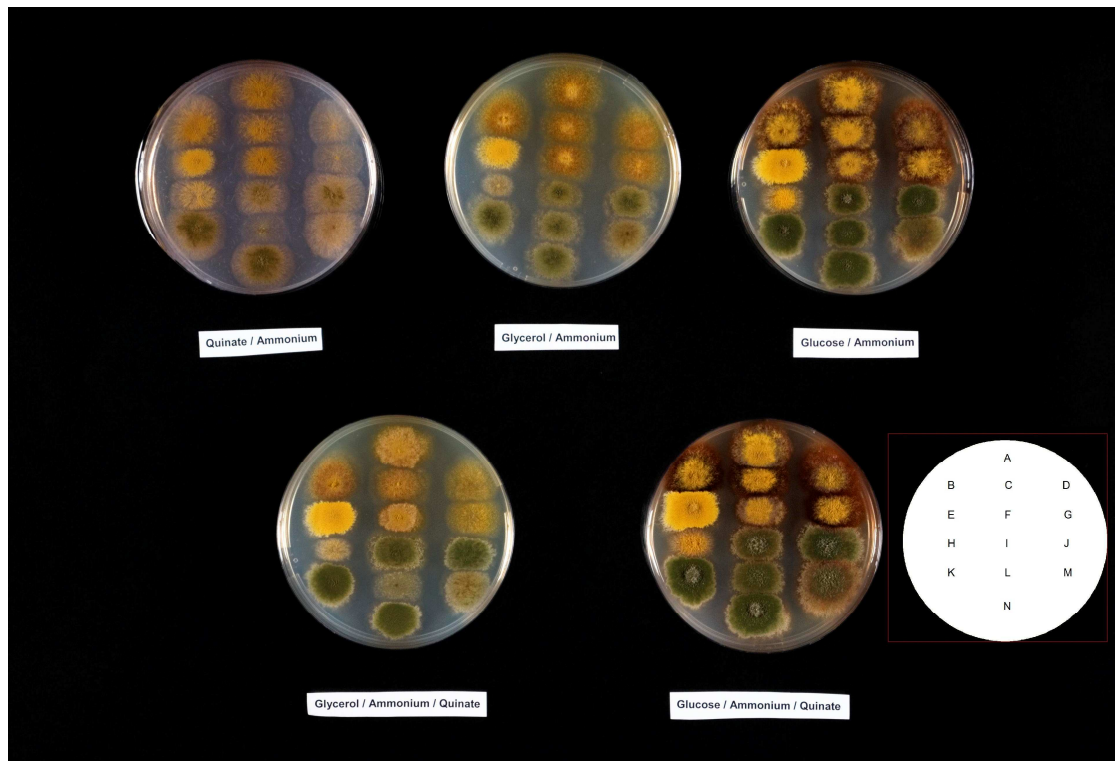


Fig 3. 1: Growth tests with 10 mM ammonium as the nitrogen source.

Both glycerol and glucose were used at a final concentration of 0.4% (w/v). Quinate was used at a final concentration of 0.1% (w/v). The carbon source combinations are: glucose (strongly catabolite repressing; transcription from the *qutE* minimal in the absence of quinate), glucose and quinate (strongly catabolite repressing, transcription from the *qutE* promoter maximal), glycerol (less strongly catabolite repressing; transcription from the *qutE* promoter minimal in the absence of quinate), glycerol and quinate (less strongly catabolite repressing, transcription from the *qutE* promoter maximal) and quinate (transcription from the *qutE* promoter maximal). A = AJC100 control, B = AJC100 transformant 21, C = AJC100 transformant 20, D = AJC100 transformant 16, E = *nmrA*Δ, F = AJC100 transformant 7, G = AJC100 transformant 24, H = *areA*Δ, I = JK2050 transformant 7, J = JK2050 transformant 8, K = JK2050 transformant 6, L = JK2050 transformant 10, M = JK2050 transformant 12, N = JK2050 control.

3.3 Growth phenotype of wild type and NmrA over-producing transformants in the presence of non-preferred nitrogen sources

The ability of transformants and controls to grow on AMM supplemented with the secondary nitrogen sources nitrate or hypoxanthine in the presence of the carbon sources described above was assessed and typical results are shown in Fig 3.2 and Fig 3.3. As *AreA* is crucial to pathways for utilising non-preferred nitrogen sources, the *areA* deletion strain failed to grow on any of the media tested. Growth of the transformants was indistinguishable from that of wild-type controls on plates lacking quinate. When growing with glucose or glycerol as carbon source transcription from the *qutE* promoter will be minimal, whereas transcription from this promoter will be maximal when growing with quinic acid as the sole carbon source. When using quinate as carbon source, most transformants showed inhibited growth phenotypes similar to that of the *areA* deletion strain, presumably as a result of overproduction of the heterologous *nmrA* from the *qutE* promoter been activated. As the *qutE* promoter is subject to carbon catabolite repression, the level of heterologous *nmrA* gene expression will remain low when using glucose and quinate in combination and will be at intermediate levels with glycerol and quinate in combination. These varying levels of transcription are predicted to produce concomitant varying levels of NmrA that will affect the growth phenotype. For example, AJC100 transformant 16 and JK2050 transformant 7 in Fig 3.2 show an extreme inhibited growth phenotype producing a minimal non-conidia colony when using quinate as carbon source. The growth phenotype is slightly less inhibited in the plate with quinate and glycerol and much less

inhibited in the plate with quinate and glucose evidenced by the increased colony radius and production of conidia.

These growth phenotypes support the hypothesis based on genetic data that NmrA acts as a repressor *in vivo*. However, this interpretation requires that the transformants showing the quinate-dependent growth inhibited phenotype on secondary nitrogen sources also have quinate-dependent elevated levels of NmrA.

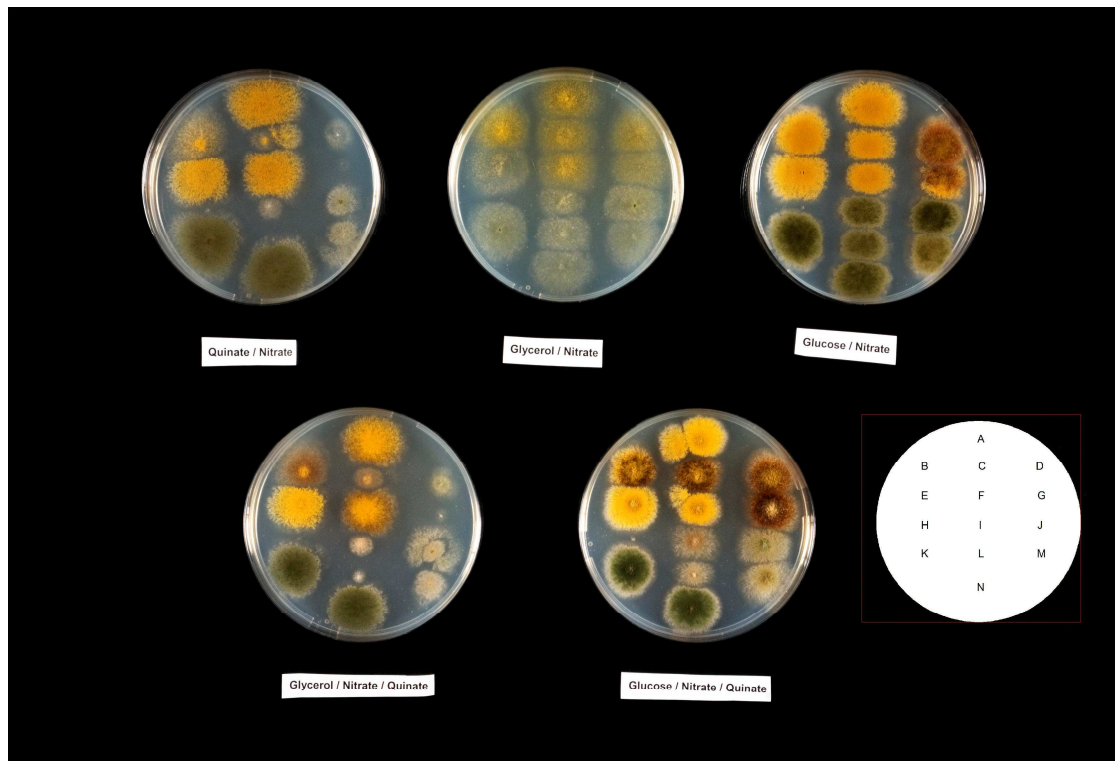


Fig 3. 2: Growth tests with 10 mM nitrate as the nitrogen source.

Both glycerol and glucose were used at a final concentration of 0.4% (w/v). Quinate was used at a final concentration of 0.1% (w/v). The carbon source combinations are: glucose (strongly catabolite repressing; transcription from the *qutE* minimal in the absence of quinate), glucose and quinate (strongly catabolite repressing, transcription from the *qutE* promoter maximal), glycerol (less strongly catabolite repressing; transcription from the *qutE* promoter minimal in the absence of quinate), glycerol and quinate (less strongly catabolite repressing, transcription from the *qutE* promoter maximal) and quinate (transcription from the *qutE* promoter maximal). A = AJC100 control, B = AJC100 transformant 21, C = AJC100 transformant 20, D = AJC100 transformant 16, E = *nmrA*Δ, F = AJC100 transformant 7, G = AJC100 transformant 24, H = *areA*Δ, I = JK2050 transformant 7, J = JK2050 transformant 8, K = JK2050 transformant 6, L = JK2050 transformant 10, M = JK2050 transformant 12, N = JK2050 control.

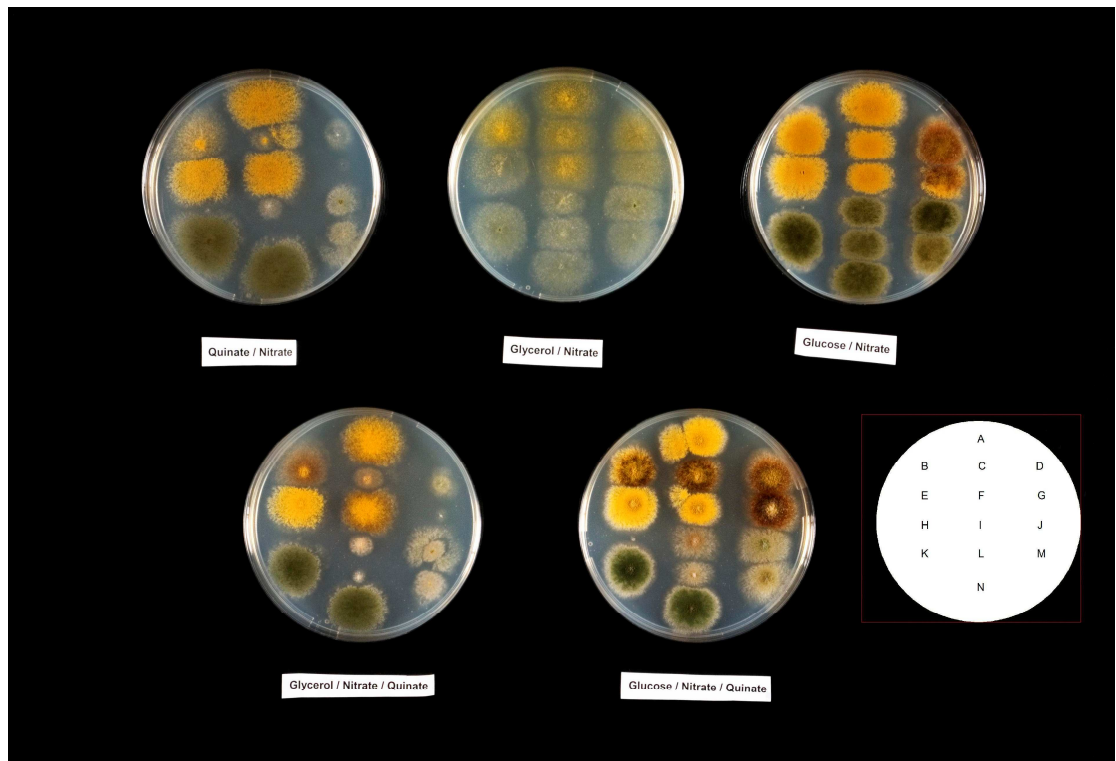


Fig 3. 3: Growth tests with 1 mM hypoxanthine as the nitrogen source.

Both glycerol and glucose were used at a final concentration of 0.4% (w/v). Quinate was used at a final concentration of 0.1% (w/v). The carbon source combinations are: glucose (strongly catabolite repressing; transcription from the *qutE* minimal in the absence of quinate), glucose and quinate (strongly catabolite repressing, transcription from the *qutE* promoter maximal), glycerol (less strongly catabolite repressing; transcription from the *qutE* promoter minimal in the absence of quinate), glycerol and quinate (less strongly catabolite repressing, transcription from the *qutE* promoter maximal) and quinate (transcription from the *qutE* promoter maximal). A = AJC100 control, B = AJC100 transformant 21, C = AJC100 transformant 20, D = AJC100 transformant 16, E = *nmrA*Δ, F = AJC 100 transformant 7, G = AJC100 transformant 24, H = *areA*Δ, I = JK2050 transformant 7, J = JK2050 transformant 8, K = JK2050 transformant 6, L = JK2050 transformant 10, M = JK2050 transformant 12, N = JK2050 control.

3.4 Increased levels of NmrA are correlated with an inhibited growth phenotype on non-preferred nitrogen sources

An NmrA-specific rabbit polyclonal antibody has previously been purified and used to probe cell free extracts of wild-type and transformant strains for their relative levels of NmrA (Johnson, 2008). The relative levels of NmrA present in cell free extracts of the transformants and control strains grown in the presence and absence of quinate were determined by western blotting. Spores of wild-type control and potential NmrA overproduction transformants were grown at 37°C for 18-19 hours, using 0.4% (w/v) glucose as carbon source and 10 mM ammonium as nitrogen source to get healthy mycelia. After washing with AMM lacking sources of carbon or nitrogen, the mycelia were transferred into fresh media containing 10 mM nitrate, 0.1% (w/v) quinate (inducing conditions), or 10 mM nitrate, 0.4% (w/v) glucose (non-inducing conditions) and grown for further 5 hours. Cell-free extracts were prepared from harvested mycelia using an extraction buffer (see Methods) that lacked the addition of a protease inhibitor cocktail. These cell free extracts were used for detecting NmrA expression by western blotting. As a control to check for quinate-dependent expression from the endogenous *qutE* promoter, duplicate blots were probed with a rabbit antibody raised against the *qutE*-encoded type II-dehydroquinase of *A.nidulans*. Probing for the type II-dehydroquinase also facilitated an assessment of the efficiency of protein transfer during the blotting process.

To normalise loadings, Lowry assays were carried out on cell free extracts to determine their protein concentrations. A total of 50 µg of protein was loaded per well, alongside

full range Rainbow Molecular Weight Markers with a molecular weight range of 10-250 kDa. Western blotting analysis was carried out as described in Methods using rabbit anti-NmrA and anti-type II dehydroquinase polyclonal antibodies (gifts from Alastair Hawkins).

Typical western blots probed with rabbit antibodies raised against NmrA or the type II-dehydroquinase are shown in Fig 3.4, where cell free extracts from transformants AJC100-16 (D in Fig 3.2) (strongly inhibited growth phenotype with quinate as carbon source) and AJC100-7 (F In Fig 3.2) (wild-type phenotype with quinate as carbon source) are analysed alongside a wild-type control strain and an *nmrA* deletion strain. Cell extracts were prepared from mycelia grown under conditions where transcription from the *qutE* promoter was minimal (glucose as carbon source) or maximal (quinat as carbon source and probed for the presence of NmrA and the type II-dehydroquinase encoded by the gene *qutE*. Inspection of Fig 3.4 shows that the type II-dehydroquinase was present in all strains grown with quinate as carbon source, thereby demonstrating that transcription from the *qutE* promoter *in vivo* had been successfully stimulated. Fig 3.4 also shows that in the duplicate blot probed with anti-NmrA antibodies no NmrA could be detected in the wild-type control and *nmrA* deletion strains implying that the levels of NmrA in the wild-type are very low. Similarly no immunoreactive species were seen in cell free extracts derived from transformant AJC100-7 implying similar low levels of NmrA *in vivo*. However, a series of strongly immunoreactive species were seen in the cell-free extracts derived from transformant AJC100-16 (strong growth inhibited phenotype with quinate as carbon source). The highest molecular weight (MW) band migrates just above the 35 kDa marker consistent with the immunoreactive protein being NmrA (predicted molecular weight of 38.9 kDa). These observations show that the growth inhibited

phenotype of transformant AJC100-16 is correlated with *in vivo* over-production of NmrA.

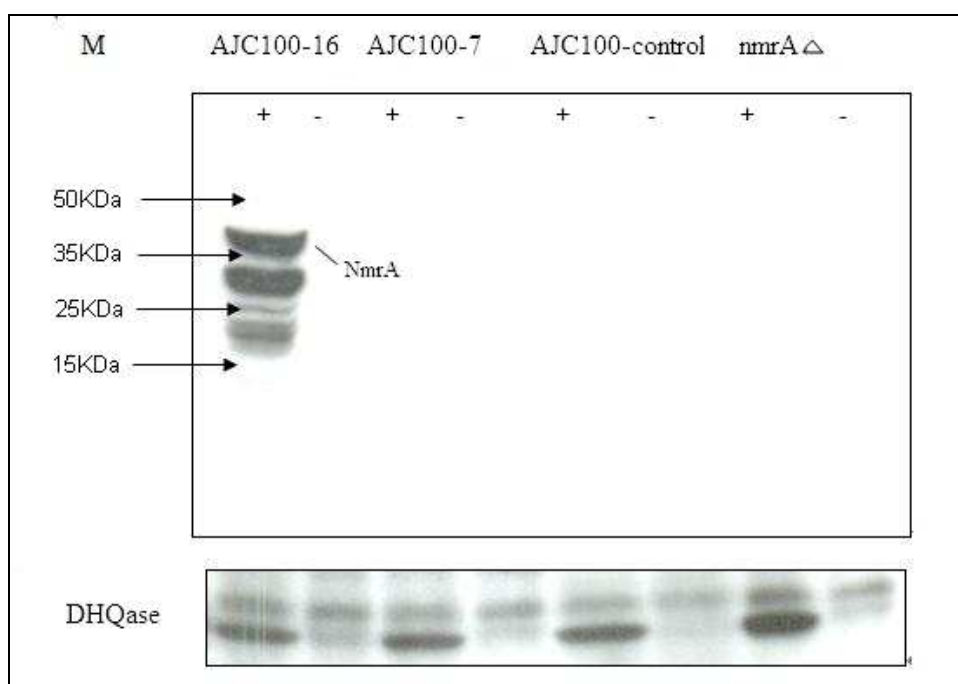


Fig 3. 4: Western blotting of NmrA (upper panel) and type-II DHQase (lower panel) of cell free extracts prepared from NmrA overproducing transformants under quinate inducing and non inducing conditions.

Spores were grown at 37°C for 18-19 hours, using 0.4% (w/v) glucose as carbon source and 10 mM ammonium as nitrogen source to get healthy mycelia. After washing with AMM lacking sources of carbon or nitrogen, the mycelia were transferred into fresh media containing 10 mM nitrate, 0.1% (w/v) quinate (inducing conditions), or 10 mM nitrate, 0.4% (w/v) glucose (non-inducing conditions) and grown for a further 5 hours. M = Markers, transformant AJC100-16 (D in Fig 3.2), transformant AJC100-7 (F in Fig 3.2), AJC100 control, nmrAΔ. +: quinate. -: no quinate. The position of NmrA is indicated.

The check for any differences that the genetic background may make on the extent of quinate-dependent NmrA overproduction, two JK2050 transformants (JK2050-10 and -12) that displayed a quinate-dependent growth inhibited phenotype on secondary nitrogen sources were analysed by western blot analysis. As shown in Fig 3.5, replicate cultures of transformants AJC100-16, JK2050-12 and JK2050-10 were grown in liquid minimal medium with either quinate or glucose as the carbon source. Cell free extracts screened for the expression level of NmrA by western blotting showed that all three transformants showed substantial quinate-dependent over-produced NmrA. This quinate-dependent NmrA overproduction correlates well with the quinate dependent inhibited growth phenotype on secondary nitrogen sources. These results suggest that the different genetic backgrounds do not have an observable effect on NmrA overproduction *in vivo* and consequently on the growth phenotype.

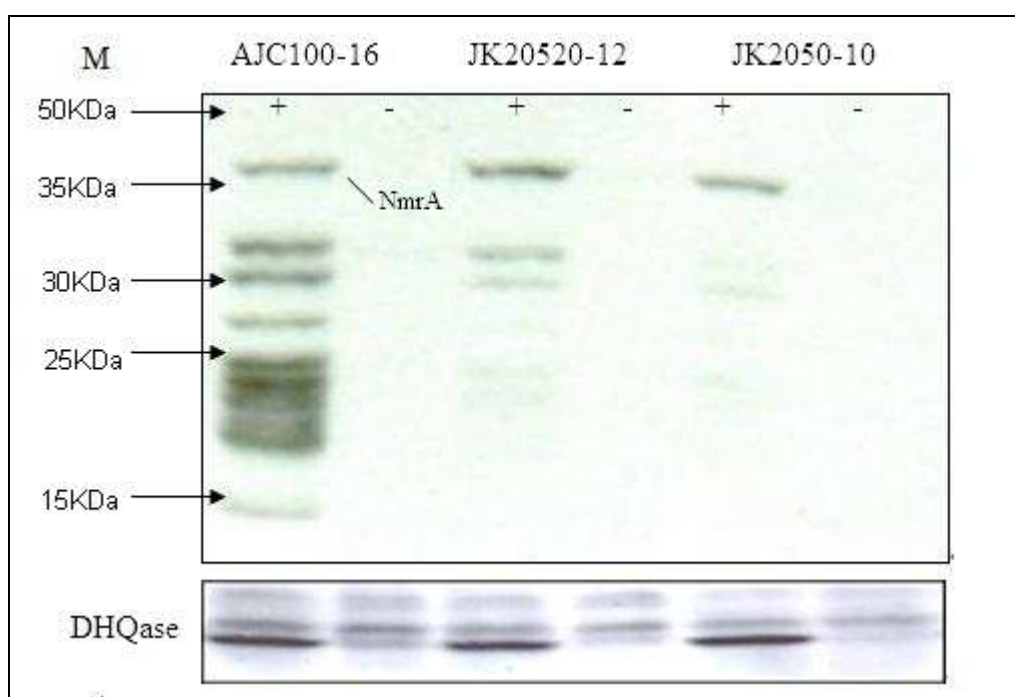


Fig 3. 5: Western blotting of NmrA (upper panel) and type-II DHQase (lower panel) of cell free extracts prepared from NmrA overproducing transformants under quinate inducing and non inducing conditions.

Spores were grown at 37°C for 18-19 hours, using 0.4% (w/v) glucose as carbon source and 10 mM ammonium as nitrogen source to get healthy mycelia. After washing with AMM lacking sources of carbon or nitrogen, the mycelia were transferred into fresh media containing 10 mM nitrate, 0.1% (w/v) quinate (inducing conditions) or 10 mM nitrate, 0.4% (w/v) glucose (non-inducing conditions) and grown for a further 5 hours. M = Markers, transformant AJC100-16 (D in Fig 3.2), transformant JK2050-12 (M in Fig 3.2), transformant JK2050-10 (L in Fig 3.2). +: quinate. -: no quinate. The position of NmrA is indicated.

Chapter 4 NmrA is subject to proteolysis

4.1 *A. nidulans* produces a proteolytic activity that digests NmrA into discrete fragments

An examination of Fig 3.4 and Fig 3.5 in chapter 3 shows that in cell free extracts of all growth inhibited transformants the overproduced NmrA was present as multiple immunoreactive bands that formed a reproducible pattern. However, control experiments analysing the type-II DHQase showed no evidence of quinate-inducible multiple immunoreactive bands. These multiple NmrA immunoreactive bands could be caused by a variety of reasons that include problems with producing or translating full length *nmrA*-specific mRNA or proteolytic degradation of full length NmrA degradation. If the latter explanation is the reason the multiple bands are produced this would imply that NmrA has some sites that are hypersensitive to proteolysis.

To initiate an analysis of the sensitivity of NmrA to proteolysis, the wild-type strain R21 was assayed for the presence of proteases using purified NmrA as the substrate. For these *in vitro* investigation, R21 spores were initially grown with 0.4% (w/v) glucose and 10 mM ammonium at 37°C for 18-19 hours to produce healthy mycelium that was subsequently washed with AMM lacking carbon and nitrogen sources. Following the wash, the mycelium was transferred into new media containing 10 mM nitrate, 0.4% (w/v) glucose (non-inducing conditions) and 10 mM nitrate, 0.1% (w/v) quinate (inducing conditions). The purpose of using the two carbon sources was to determine if the mycelium produced the proteolytic activity in response to the presence of quinate. As the endogenous level of wild type NmrA in the cell free extracts was too low for detection, purified NmrA protein (60 ng per well) was used as the substrate.

Purified NmrA was incubated at 25°C with the corresponding cell free extracts in the presence and absence of a proteases inhibitor cocktail (30 ng per well). 12 µl samples were withdrawn from the digestion mixtures at intervals of 0, 15 mins, 30 mins, 45 mins, 60 mins, 90 mins, 120 mins and 180 mins and proteins separated by SDS-PAGE in preparation for western blot analysis.

Typical western blotting results (Fig 4.1 and Fig 4.2) show that *in vitro* the degradation of NmrA occurs when mixed with cell free extracts of mycelium grown in the presence of quinate or glucose. Furthermore, the proteolytic degradation of NmrA was inhibited by the addition of a protease inhibitor cocktail. The type-II DHQase remained stable during incubation. These observations show that the production of the proteolytic activity in *A. nidulans* mycelium is not quinate dependent. The majority of NmrA protein was digested into two major smaller fragments within 15 mins, implying that NmrA has sites that are hypersensitive to the presence of the proteolytic activity.

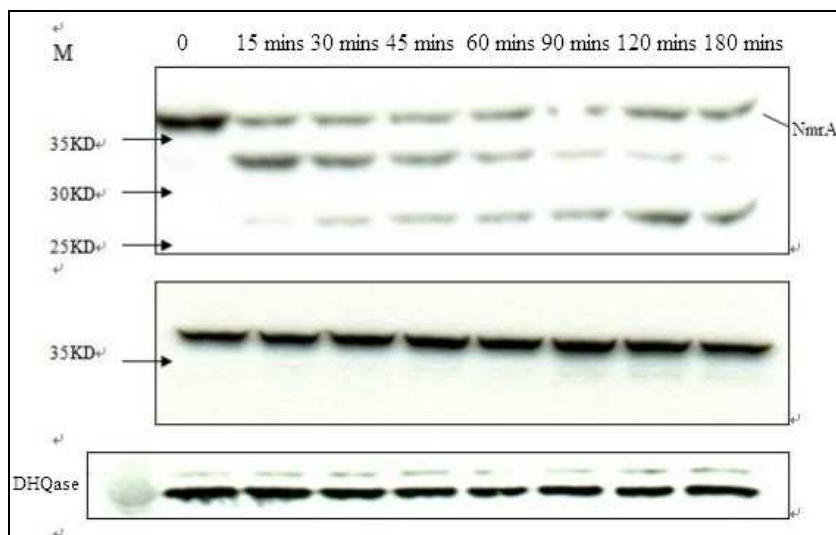


Fig 4. 1: Western blotting of NmrA and type II DHQase incubated with cell extracts from mycelia grown with quinate as the carbon source.

Upper panel: purified NmrA incubated with cell extracts (no protease inhibitors). Middle panel: purified NmrA incubated with cell extracts (protease inhibitors added). Lower panel: type-II DHQase incubated with cell extracts (no protease inhibitors). M = Markers, Lanes 1-8 = incubation for 0, 15 mins, 30 mins, 45 mins, 60 mins, 90 mins, 120 mins, 180 mins. The position of NmrA is indicated.

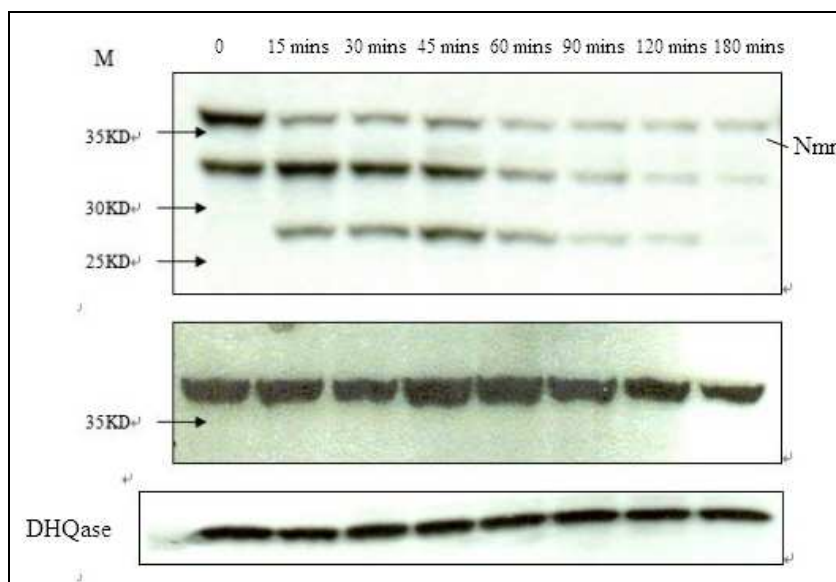


Fig 4. 2: Western blotting of NmrA and type II DHQase incubated with cell extracts from mycelia grown with glucose as the carbon source.

Upper panel: purified NmrA incubated with cell extracts (no protease inhibitors). Middle panel: purified NmrA incubated with cell extracts (proteases inhibitors added). Lower panel: type-II DHQase incubated with cell extracts (no protease inhibitors). M = Markers, Lanes 1-8 = incubation for 0, 15 mins, 30 mins, 45 mins, 60 mins, 90 mins, 120 mins, 180 mins. The position of NmrA is indicated.

4.2 Proteolysis assay development

The western blot assay used to detect the proteolysis of NmrA proved to be variable and of limited use in comparing the relative levels of proteolytic activity in independently prepared cell free extracts. To alleviate this problem the level of NmrA in the assay was increased from 60 ng to 2 μ g so that the digestion products of NmrA could be visualised after separation by SDS-PAGE and staining for protein with Coomassie Blue R250. A typical acrylamide gel showing Coomassie stained protein is shown in Fig 4.3. In this example multiple samples of 2 μ g purified NmrA were incubated at 25°C with 1 μ g each of cell free extracts in 50 mM potassium phosphate buffer, pH 7.2 at a final volume of 10 μ l for time periods in the range 30 mins to 300 mins. This method proved to be highly reproducible and was used in all succeeding proteolysis assays.

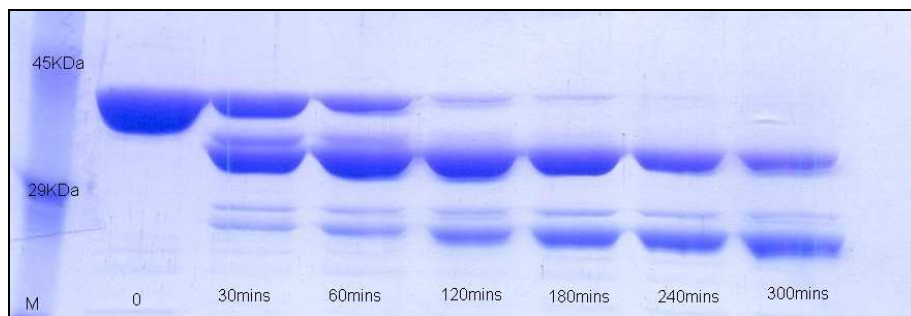


Fig 4. 3: NmrA proteolysis assay for cell free extracts of wild type R21.

The sample is the same as that shown in Fig 4.2 and is prepared from the mycelium grown with glucose as the carbon source. 1 μ g of cell free extracts were incubated at 25°C with 2 μ g purified NmrA in 50 mM phosphate buffer, pH 7.2 at final volume of 10 μ l for each sample running on the gel. Time periods are ranged from 0, 30 mins, 60 mins, 120 mins, 180 mins, 240 mins to 300 mins.

4.3 pH changes are not correlated with protease production

In order to determine the factor(s) affecting protease production within *A. nidulans* mycelium, several potential parameters were considered. It was unknown if protease production was constitutive or if it was stimulated in response to some metabolic signal(s). Such signals could include the nature of the nitrogen source (primary or secondary nitrogen source) or the concentration of the nitrogen source. When mycelium is actively growing the concentration of carbon and nitrogen sources will fall and in a liquid fermentation the pH of the growth medium is likely to change as a result of excretion of waste products and depletion of buffer salts during growth. Therefore it was considered necessary to investigate if starvation for carbon source or nitrogen source or pH change could act as the metabolic signal(s) for protease production.

The possible involvement of pH was investigated initially by determining the extent of any pH changes associated with growth under the standard conditions described in methods. The amounts of 2×10^8 spores were initially grown at 37°C in 200 ml medium with 0.4% (w/v) glucose and 10 mM ammonium for 19 hours. The pH of the standard liquid AMM used in these experiments is 6.6, and the pH at the end of the 19-hour fermentation at 37°C was measured in replicate cultures. The results are summarised in Table 4.1, inspection of which shows that the pH of the growth medium changes from 6.6 to an average value of 8.16 after 19 hours growth at 37°C and the average pH change is around 1.56. The effect of this pH change was investigated by growing mycelium in standard medium at pH 6.6, and then transferring 50% of the mycelium into new medium at pH 6.6 and 50% into the medium with its pH adjusted to

correspond to the pH of the original growth medium at the end of the initial 19 hour growth period. After the initial 19 hours growth the mycelia were washed with and then transferred into the corresponding new medium for a further 5 hours growth. This experiment was carried out 5 times and in two cases an additional growth condition, at pH 4.5, was included to eliminate the differences between alkaline pH and acid pH (the mycelia were equally divided and transferred into three new media with different pHs). Cell free extracts prepared from the mycelia harvested after final pH measurement were assayed for the production of proteases using NmrA as the substrate (see Table 4.1). In all five data sets, no proteolytic activity was apparent in the mycelia that were transferred into media that had their pH adjusted to match that at the end of the 19 hour initial growth period. This observation implies that the gradual pH change of the media caused by mycelia growth does not stimulate protease production. The two sets of cell free extracts showing proteolytic activity were derived from mycelia that had been subject to an average pH shock of 1.6 units. This raises the possibility that sudden pH change associated with transfer to new medium may stimulate protease production. However, the highest pH change at 1.74 was from set ③ which had no apparent proteolytic activity. Additionally, after 19 hours growth mycelia that been transferred into media of widely differing pH from 4.5 to 8.3 (as shown in set ③ and ④), showed no apparent protease activity. In summary, these data show that that exposure to gradual or sudden pH change is not associated with the production of proteases.

Set	①		②		③		④			⑤			Average
Starting pH	6.6		6.6		6.6		6.6			6.6			6.6
pH after 19 hours	8.28		7.9		8.34		8.11			8.16			8.16
pH change after 19 hours	1.68		1.3		1.74		1.51			1.56			1.56
pH of new media	6.6	8.28	6.6	7.9	6.6	8.34	6.6	8.11	4.5	6.6	8.16	4.5	
Final pH	6.14	7.1	6.18	6.9	6.08	7.01	6.13	7.09	4.96	6.08	7.07	5.03	
Final pH change	0.46	1.18	0.42	1.0	0.52	1.23	0.47	1.02	0.45	0.52	1.09	0.53	
Proteolysis	+	-	-	-	-	-	-	-	-	+	-	-	

Table 4. 1: pH measurement of media of before beginning and after of growth mycelial growth.

Spores were grown at 37 °C for 19 hours and then transferred into media of different pH for a further 5 hours. Cell free extracts after final growth were screened by the NmrA proteolysis assay. +: protease activity detected. -: protease activity not detected.

4.4 Nitrogen limitation stimulates maximal protease production

To determine if the nature (primary, secondary or neutral) or the concentration of nitrogen source in the growth medium affected protease production, ammonium, nitrate, hypoxanthine and proline were screened in liquid medium growth tests. R21 spores were grown in AMM with 0.4% (w/v) glucose and 10 mM ammonium at 37°C for 18-19 hours to produce mycelia. After washing with AMM lacking sources of nitrogen and carbon, mycelia were transferred into new media containing 0.4% (w/v) glucose as carbon source, and either 10 mM ammonium, 10 mM nitrate, 10 mM hypoxanthine or 10 mM proline as individual nitrogen sources for a further 5 hours growth. Cell free extracts from all the harvested mycelia were then screened in the NmrA proteolysis assay, and the results are shown in Fig 4.4. Inspection of Fig 4.4 shows that transfer of mycelia grown on ammonium into fresh medium containing a primary nitrogen source (ammonium), a secondary nitrogen sources (nitrate, or hypoxanthine) or a neutral nitrogen source (proline) all resulted in the production of proteolytic activity. The presence of the proteolytic activity in all conditions implies that production of the proteases is not dependent on the nature of the nitrogen source. However, these experiments do not rule out the possibility that the mechanical disruption associated with harvesting and transferring the mycelia to fresh medium may be the signal for protease production. To investigate this possibility R21 spores were grown at 37°C for 19 hours in media with 10 mM ammonium or nitrate as nitrogen source and then washed with and transferred into new equivalent media for a further 5 hours growth. Cell free extracts were prepared from samples harvested before and

after transferring to fresh equivalent media and screened for proteolytic activity. As shown in Fig 4.5, proteolytic activity was present in all mycelium screened, confirming the results in Fig 4.5 and showed that the proteolytic activity was present in cell free extracts prepared from mycelium before its transfer to new medium. Except in the unlikely event that protease production is instantaneous, these observations imply that the proteolytic activity was present in the mycelium after the initial 19 hours growth.

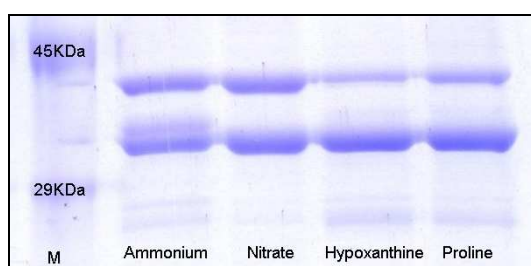


Fig 4. 4: NmrA proteolysis assay for cell free extracts of mycelia after grown with ammonium transferred into media with different nitrogen sources.

Mycelia (R21) were grown utilising 0.4% (w/v) glucose and 10 mM ammonium for 19 hours at 37°C before transferring into new media with 10 mM ammonium/ nitrate/ hypoxanthine/ proline for a further 5 hours growth. 1 µg of cell free extracts were incubated with 2 µg purified NmrA protein at 25°C for 30 mins. The positions of 29 kDa and 45kDa markers are indicated.

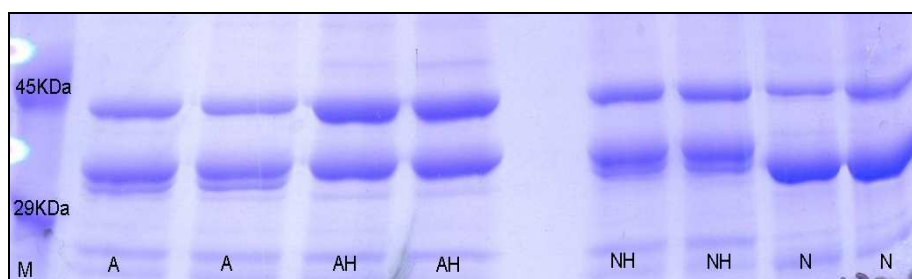


Fig 4. 5: NmrA proteolysis assay for cell free extracts of mycelia utilising ammonium or nitrate as nitrogen source both before and after transferring.

Mycelia were grown utilising 0.4% (w/v) glucose and 10 mM ammonium/nitrate for 19 hours at 37°C before transferring into new equivalent media for a further 5 hours growth. AH: mycelia utilising ammonium before transferring. A: mycelia utilising ammonium after transferring. NH: mycelia utilising nitrate before transferring. N: mycelia utilising nitrate after transferring. 1 µg of cell free extracts were incubated with 2 µg purified NmrA protein at 25°C for 30 mins. The positions of 29 kDa and 45 kDa markers are indicated.

To test the hypothesis that nitrogen insufficiency may be the signal that stimulates proteolytic activity, R21 spores were grown at 37°C for 18-19 hours in minimal media containing 5 mM, 10 mM, 20 mM or 40 mM ammonium or nitrate as nitrogen source, and 0.4% (w/v) glucose was used as carbon source. After this growth period cell free extracts were screened for the production of proteolytic activity using NmrA as the substrate.

The extent of proteolysis varied as shown in Fig 4.6. The protease activity was consistently stimulated by growth in lower concentration of nitrogen and consistently absent in mycelia grown in high (40 mM) concentrations of nitrogen. To further test this apparent correlation, cell free extracts of mycelium grown in minimal medium containing 2.5 mM, 5 mM, 10 mM, 20 mM or 40 mM ammonium were screened for proteolytic activity using NmrA as the substrate. Seven data sets were analysed this way and the data are summarised in Table 4.2. Inspection of Table 4.2 shows that in all cases the production of proteases detectable with NmrA as the substrate was associated with growth in the lower concentrations of nitrogen.

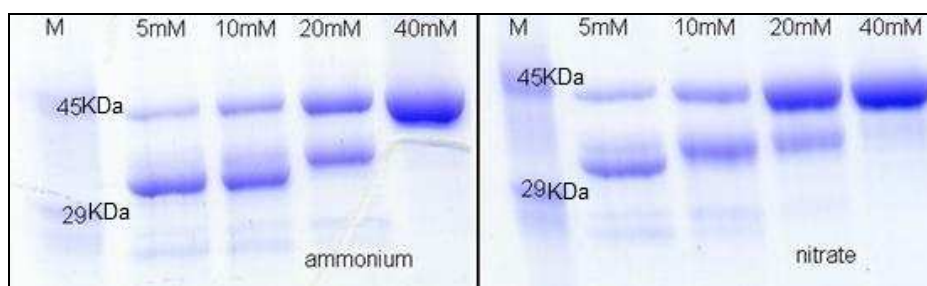


Fig 4. 6: Proteolysis assay of the cell free extracts from mycelia growing with different concentration of nitrogen source.

Spores of wild type strain R21 were grown with 0.4% (w/v) glucose as carbon source and ammonium tartrate/ sodium nitrate as nitrogen sources at 5 mM, 10 mM, 20 mM and 40 mM concentration. Mycelia were harvested after 19 hours growth at 37°C and 1 µg of cell free extracts were incubated with 2 µg purified NmrA protein at 25°C for 30 mins. The positions of 29 kDa and 45 kDa markers are indicated.

Set	2.5 mM	5 mM	10 mM	20 mM	40 mM	Nitrogen source
①	+	+	+	+	-	Ammonium
②	+	+	+	+	-	Ammonium
③	+	+	+	+	-	Ammonium
④	+	+	-	-	-	Ammonium
⑤	+	-	-	-	-	Ammonium
⑥	+	-	-	-	-	Ammonium
⑦	+	+	-	-	-	Nitrate

Table 4. 2: Proteolysis assay for mycelia grown with different concentrations of nitrogen source.

Spores of wild type strain R21 were grown with 0.4 % (w/v) glucose at 37 °C for 19 hours before harvest, using 2.5 mM, 5 mM, 10 mM, 20 mM and 40 mM ammonium or nitrate as nitrogen source. 1 µg cell free extracts were incubated with 2 µg purified NmrA protein at 25 °C for 30 mins. +: positive in NmrA digestion. - : negative in NmrA digestion.

These observations imply that the signal for protease production is present in nitrogen limiting conditions. To test this hypothesis directly cell free extracts were prepared from mycelium grown at 37°C for 19 hours in minimal medium with 40 mM ammonium as nitrogen source and then transferred to and incubated for 5 hours in minimal medium lacking nitrogen. The results of this experiment are shown in Fig 4.7 where it can be seen that proteolytic activity is only present in cell free extracts of mycelium that have been starved of nitrogen. This observation is consistent with the interpretation that the signal for the production of the proteases detected using NmrA as the substrate is growth in nitrogen limiting or nitrogen starvation conditions.

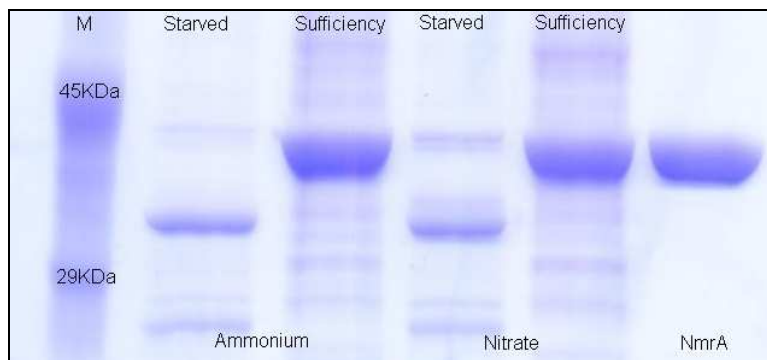


Fig 4. 7: Proteolysis assay of the cell free extracts from mycelia growing under nitrogen sufficiency and nitrogen starvation conditions.

Spores of wild type strain R21 were grown with 0.4% (w/v) glucose as carbon source and ammonium / nitrate as nitrogen sources at 40 mM (nitrogen sufficiency conditions). Mycelia were then transferred into minimal medium lacking nitrogen (nitrogen starved conditions) nitrogen starvation condition for another 5 hours before harvest. 1 µg cell free extracts were incubated with 2 µg purified NmrA protein at 25°C for 30 mins. Purified NmrA was used as undigested control. The positions of 29 kDa and 45 kDa markers are indicated.

4.5 Protease production is independent of the carbon source

The data in the preceding section show that exposure to nitrogen limiting conditions is the likely cause of the production of proteases detected when using NmrA as the substrate. Exposure to nitrogen limiting conditions can come about by exhausting the nitrogen supply due to mycelial growth, however, if this is the case then it is likely that the supply of carbon will also become limiting. To test the possibility that carbon limitation may also influence the production of proteases detectable using NmrA as the substrate, mycelium was grown under a range of carbon and nitrogen regimes. R21 spores were grown at 37°C for 18-19 hours under four conditions: Both carbon and nitrogen limitation (0.1% (w/v) glucose, 2.5 mM ammonium) ; carbon limitation (0.1% (w/v) glucose, 40 mM ammonium); nitrogen limitation (0.4% (w/v) glucose, 2.5 mM ammonium) and carbon and nitrogen sufficiency conditions (0.4% (w/v) glucose, 40 mM ammonium). Cell free mycelial extracts were assayed for protease production using NmrA as the substrate and the results are shown in Fig 4.8. Inspection of Fig 4.8 shows that under conditions of glucose limitation and nitrogen sufficiency the protease assay was negative whereas under the control conditions of glucose sufficiency and nitrogen limitation the protease assay was positive. These observations imply that carbon limitation is not influencing the production of proteases detectable using NmrA as the substrate.

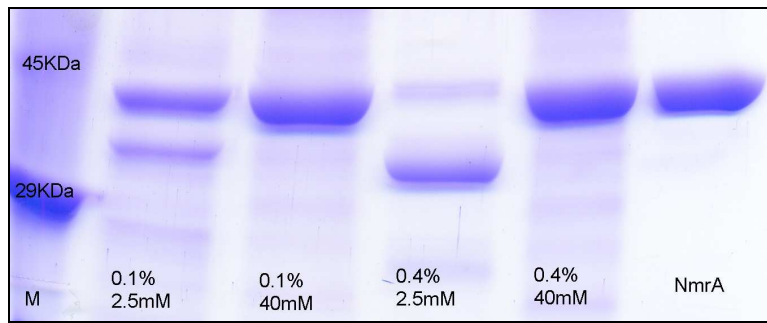


Fig 4. 8: Proteolysis assay for combination conditions of carbon and nitrogen limitations.

Spores of wild type strain R21 were grown with 0.1% (w/v) glucose, 2.5 mM ammonium; 0.1% (w/v) glucose, 40 mM ammonium; 0.4% (w/v) glucose, 2.5 mM ammonium; 0.4% (w/v) glucose, 40 mM ammonium at 37°C for 19 hours before harvest. 1 µg Cell free extracts were incubated with 2 µg purified NmrA protein at 25°C for 30 mins. Purified NmrA was used as undigested control. The positions of 29 kDa and 45 kDa markers are indicated.

4.6 Kinetics of protease production

In order to help characterise the induction kinetics of protease production, a casein assay was adopted in addition to the assay using NmrA as the substrate for measuring the protease activity. The commercially available E6638 *EnzCheck* Protease Assay kit was used for the fluorescent assay as the casein substrate is commonly used as a non-specific protease substrate. The purpose of using the casein substrate was to facilitate a more sensitive and quantitative measure of protease activity. The casein assay was set up as follows: 5 µg and 10 µg amounts of protein from cell free extracts were incubated with 10 µg of the casein substrate in 50 mM phosphate buffer, pH 7.2 at a final volume of 1 ml at room temperature (20°C). Fluorescent readings were taken every minute up to ten minutes and one unit of protease activity was defined as a fluorescence change of 100 units per minute.

At first, in order to see how the sensitivity of this casein assay compared to the NmrA proteolysis assay, 10 µg of protein from cell free extracts prepared from mycelium grown in 2.5 mM ammonium/ nitrate (nitrogen limitation condition) and 40 mM ammonium/ nitrate (nitrogen sufficiency condition) were analysed in both proteolysis assays. The NmrA proteolysis results were the same as those shown in Fig 4.7, with proteolysis present at low concentration of ammonium and absent at high concentration of ammonium. The casein assay results in Fig 4.9 show a similar pattern with protease activity present in cell free extracts of mycelium grown in low concentrations of ammonium and protease activity absent in mycelium grown at high ammonium concentrations. One unit (U) of the protease activity was defined as 100 fluorescence units (FUs) in one minute. The protease activity of cell extracts from the mycelia

grown with 2.5 mM ammonium was 12 U mg^{-1} and the protease activity of cell extracts from the mycelia grown with 2.5 mM nitrate was 9.8 U mg^{-1} . A comparison of Fig 4.8 and Fig 4.9 shows that the casein assay and the NmrA-based proteolysis assays were very similar and complemented each other in assaying for the presence of proteases.

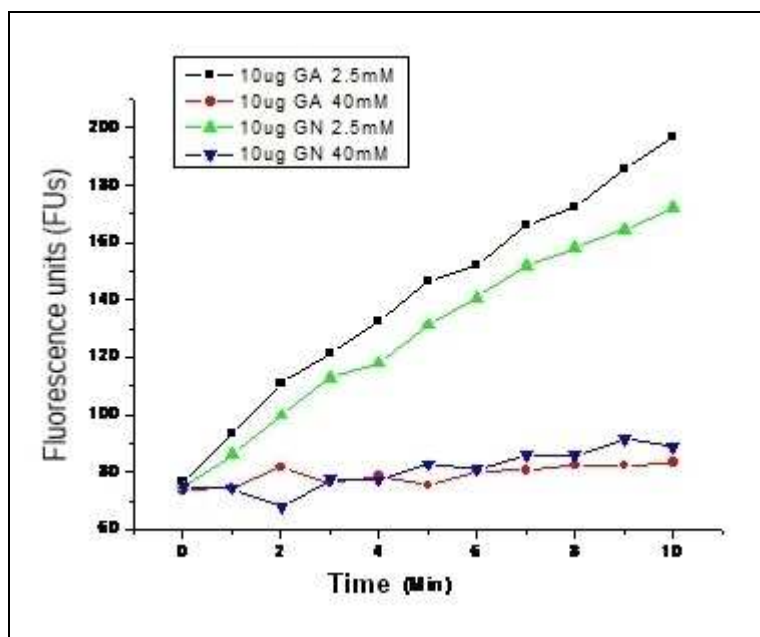


Fig 4. 9: Casein assay for samples under nitrogen limitation condition and nitrogen sufficiency condition.

Spores of wild type strain R21 were grown at 0.4% (w/v) glucose (G), 2.5 mM ammonium (A)/ nitrate (N) or 40 mM ammonium/ nitrate at 37°C for 19 hours before harvest. X scale: Time changes (min). Y scale: fluorescence unit changes (Fu).

To further investigate the kinetics of protease production in response to nitrogen starvation, R21 spores were grown at 37°C for 18-19 hours in media with 0.4% (w/v) glucose and 40 mM ammonium and, following a wash with nitrogen free medium, were transferred into nitrogen free medium and replicate cultures incubated for 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 16 hours and 24 hours. Cell free extracts of mycelia from these cultures were tested in both the NmrA-based proteolysis assay and in the casein assay. The experiment was carried out five times and the data are summarised in Table 4.3.

Activity Time U mg^{-1} Set	0 h	2 h	4 h	6 h	8 h	10 h	16 h	24 h
①	0	0	10.4	9.0	11.9	11.3	N/A	12.0
②	0	0	0	2.5	6.4	7.4	N/A	8.3
③	0	0	0	6.6	7.9	6.1	6.0	8.4
④	0	0	0	7.3	8.5	5.5	5.5	6.0
⑤	0	0	7.5	8.5	8.8	10.2	9.4	9.6

Table 4. 3: Casein assay for protease activities under nitrogen starvation conditions.

Spores of wild type strain R21 were grown with 0.4% (w/v) glucose and 40 mM ammonium at 37°C for 19 hours and then washed and transferred into new media utilising 0.4% (w/v) glucose with no nitrogen source, starved for 0, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 16 hours, 24 hours before harvest.

Fig 4.10 and Fig 4.11 show the data from set ③ and are a typical of all the experiments. Inspection of Fig 4.10 and Table 4.3 shows several features of interest. First, the NmrA-based protease assay appears to be more sensitive than the casein assay as it detects protease activity in cultures starved of nitrogen for two hours. This contrasts to the casein assay which fails to detect protease activity at two hours in any of the five repeat experiments. These observations imply that either the NmrA-based proteolysis assay is more sensitive than the casein-based assay, or that a succession of proteases is being produced and that the casein is not acting as substrate for them all. Second, the fluorescent assay demonstrates that protease levels between cultures follow a similar trend of increasing activity with time as revealed by the NmrA-based assay. However the trend is somewhat variable and points to other factors that may be influencing the observed result. These factors will include variations within the microenvironment within each of the 2 L flasks used for each growth experiment. Although a common stock of medium was used throughout any one set of mycelial growths, factors such as differences in the extent of baffling introduced into the flasks to aid aeration and differences in the extent to which the growing mycelium can colonise the side of the flask during growth will all have effects on the rate of growth and access to the nitrogen in the growth medium. To try and minimise these effects, the same set of flasks, with the same baffling pattern, was used throughout the experiments described above.

In summary the data described above show that the production of proteases in response to nitrogen starvation begins within two hours and that the NmrA has sites that are hypersensitive to digestion.

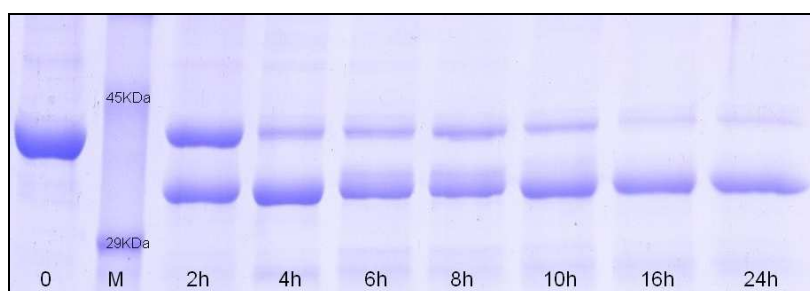


Fig 4. 10: NmrA proteolysis assay with cell free extracts of wild type R21 prepared from mycelia exposed to nitrogen starvation conditions for different times.

Spores of wild type strain R21 were grown at 0.4% (w/v) glucose and 40 mM ammonium at 37°C for 19 hours before washed and transferred into new media without nitrogen sources for 0, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 16 hours and 24 hours before harvest.

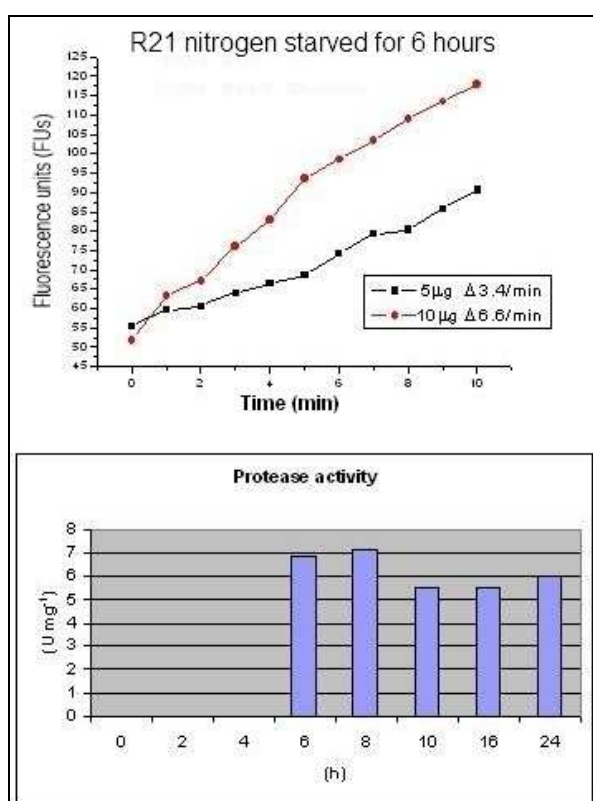


Fig 4. 11: Protease activity of R21 cell free extracts of mycelium exposed to nitrogen starvation condition for different times.

Spores of wild type strain R21 were grown in 0.4% (w/v) glucose and 40 mM ammonium at 37°C for 19 hours before washing and transferring to new media without nitrogen sources for 0, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 16 hours and 24 hours before harvest (set ③ from Table 4.3). Upper panel: Casein assay of the cell free extracts starved for 6 hours. Lower panel: protease activity of cell free extracts starved from 0 hours up to 24 hours. The protease activity is detectable after starved for 6 hours.

Chapter 5 *A. nidulans* has three NmrA-hypersensitive protease activities

5.1 Chromatographic separation of proteases from cell-free extracts of wild-type *A. nidulans*

In order to purify and characterise the proteases produced in response to nitrogen starvation, the proteins present in cell free extracts of nitrogen starved mycelia were subjected to ion exchange chromatography as described in Methods. Fractions eluted by salt gradient from the DEAE column were assayed in the NmrA-based proteolysis assay and typical results are shown in Fig 5.1. Inspection of Fig 5.1 shows that this procedure demonstrated the presence of two separable peaks of protease activity. One peak of activity, designated PNM1A, washed through the column without binding while the other, designated PNM1B, bound to the column and was eluted by around 0.5 M NaCl. Inspection of Fig 5.1 and Fig 5.2 shows that both protease peaks produced major proteolysis fragments that were similar but distinct sizes that correspond to the fragments seen when non-chromatographed samples are assayed (as shown in Fig 4.3). The major fragment produced by PNM1A was designated NM1A and the major fragment produced by PNM1B was designated NM1B (Fig 5.1).

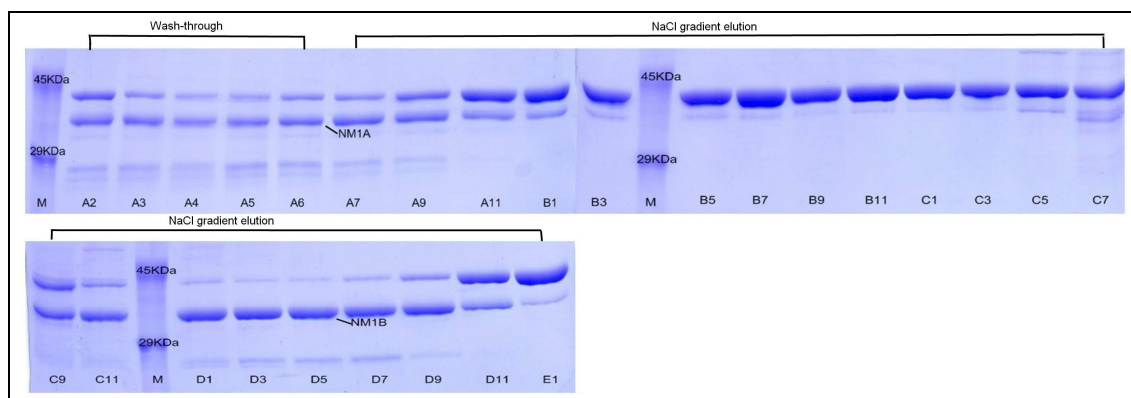


Fig 5. 1: The separation of proteolytic activities by chromatography on a 5 ml Hi Trap DEAE FF column.

10 μ l of each fraction were screened in the NmrA-based proteolysis assay. Fractions A2-A6 are the 10 ml wash through fractions. Fractions A7-E1 are the 2 ml fractions eluted by the 0.0-1.0 M NaCl gradient. Fragment Nm1A was washed off the column first and fragment Nm1B was eluted off around 0.5 M NaCl. The positions of 29 kDa and 45 kDa markers are indicated.

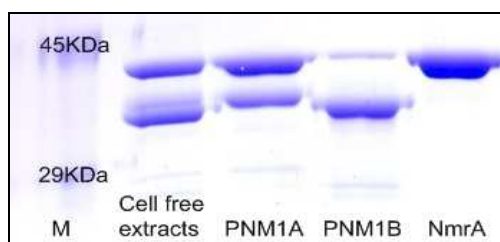


Fig 5. 2: NmrA-based proteolysis assay with crude and chromatographically separated cell free extracts containing PNM1A and PNM1B.

1 μ g of crude cell free extracts of R 21 and 10 μ l volumes of fractions containing PNM1A and PNM1B (fractions A3 and D1 from Fig 5.1) following DEAE FF chromatography were incubated with 2 μ g purified NmrA at 25°C for 30 mins, respectively. Purified NmrA was used as an undigested control. The positions of 29 kDa and 45 kDa markers are indicated.

5.2 Identification of two proteases by LC-MS

In order to further purify and try to identify the PNM1A and PNM1B, fractions containing each protease were further separated by Superdex column. Eluted fractions were screened in the NmrA-based proteolysis assay and active fractions concentrated by either TCA precipitation or Vivaspin centrifugal concentration (see Methods). After the concentration step, the protein content of the samples was examined by running on both 10% and 15% SDS-PAGE gel. Samples containing PNM1A showed multiple weakly staining bands (see Fig 5.3), and these were excised from the gel using a scalpel blade in preparation for identification by peptide mass spectrometry and LC-MS. The results of this analysis are shown in Table 5.1, inspection of which shows that of the six proteins identified only one (Q5BGU2_EMENI, Mascot score= 219 in MSDB database) was a protease (Fig 5.4). The protease identified was a subtilase serine protease of predicted MW of 89.8 kDa, encoded by the gene AN0238.2. However, the estimated MW of the protein in the SDS-PAGE gel is between 29 kDa and 45 kDa, which do not match with the MW of the protein encoded by the gene AN0238.2. Subtilase proteins are known to be processed from a larger inactive form into a smaller active form therefore the difference in measured and predicted MW does not necessarily cause a problem with the accurate identification of this protein (Siezen and Leunissen, 1997). This procedure was repeated two times and in each case the only protease was identified as subtilase. However, the MW difference is a caveat that must be borne in mind when interpreting the data.

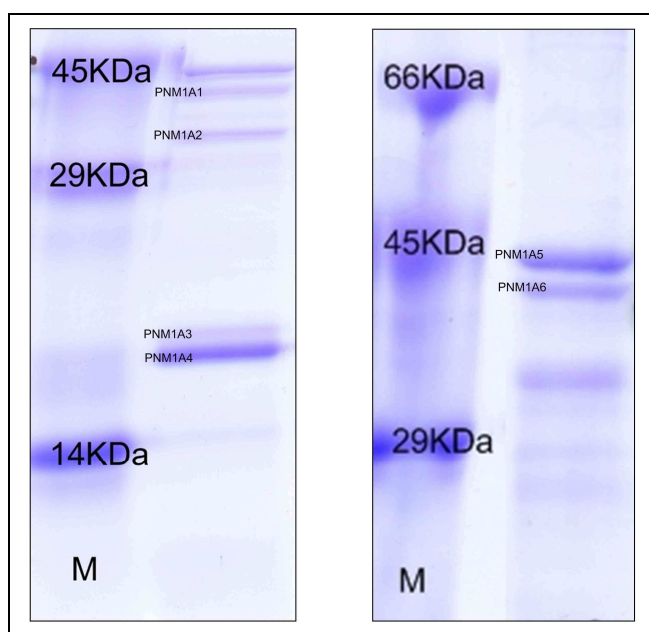


Fig 5. 3: SDS-PAGE gel electrophoresis of PNM1A containing fractions from Superdex column.

Left: fraction running on a 15% SDS-PAGE gel. Right: fraction running on a 10% SDS-PAGE gel. Bands labelled as PNM1A1, PNM1A2, PNM1A3, PNM1A4, PNM1A5, PNM1A6 were excised for LC-MS.

Num	Name	Result	Reference
PMS1	PNM1B	Q5BAR4_EMENI	Hypothetical protein: Trypsin-like protease MW = 25434 Da
PMS2	PNM1Bp2	Q5BAR4_EMENI	Hypothetical protein: Trypsin-like protease MW = 25434 Da
PMS3	PNM1A1	Q7Z8L1_EMENI	NADP(+)-dependent glycerol dehydrogenase MW = 36958 Da
PMS4	PNM1A2	Q5BGU2_EMENI	Hypothetical protein subtilase protease MW = 89800 Da
PMS5	PNM1A3	SODC-EMENI	Superoxide dismutase [Cu-Zn] MW = 15847 Da
PMS6	PNM1A4	Q5ASX5_EMENI	Proline isomerase MW = 17787 Da
PMS7	PNM1A5	Q92222_EMENI	Chitinase MW = 44178 Da
PMS8	PNM1A6	Q5AUW6_EMENI	Zn-binding protein MW = 35950 Da

Table 5. 1: Peptide mass spectrometry and LCMS-MS results for identification of PNM1A and PNM1B.

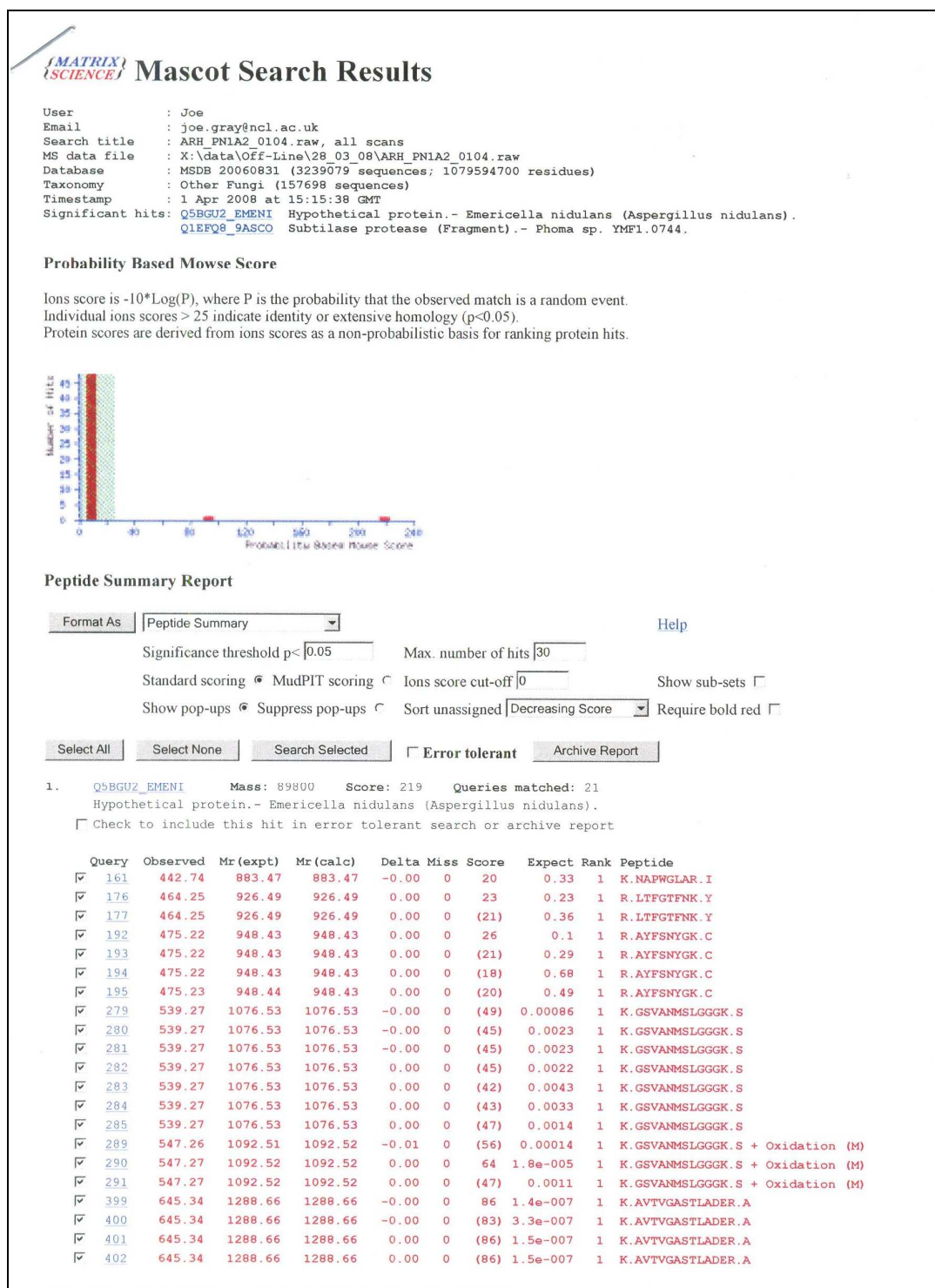


Fig 5. 4: Mascot identity searching analysis of PNM1A.

The MASCOT score for Q5BGU2_EMENI is 219 and scores greater than 25 are significant at the 95% confidence limit. The MASCOT score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. The bar chart represents the significance of the recognised protein. The top scoring peptide matches for the spectrum are shown in red.

Unlike the situation with PNM1A, there was only a single strongly staining band in the sample containing PNM1B. The peptides generated by tryptic digest were analysed by two independent means, Mascot identity database searching and BLAST homology searching. The Mascot analysis (Fig 5.5) showed that several digest peptides matched significantly with the hypothetical protein Q5BAR4_EMENI from *A. nidulans* (Mascot score = 252; threshold score = 25; probability of a random match = 1 in 10²² using the MSDB database). An independent analysis method using the program DeNovoX was also used to attempt to sequence the peptides *de novo* from MS/MS spectra. This is without prior reference to any databases. The results of this analysis were manually inspected and the best 25-30 peptide MS/MS sequences were extracted to a list for BLAST homology searching in both directions. The results of the BLAST search (Fig 5. 6) showed that the only significant matching protein from searching the entire Swiss_Prot database identified the same hypothetical sequence as with Mascot. This match also provided extra sequence coverage while giving added confidence in the protein identification by an independent means of analysis. Q5BAR4_EMENI is a hypothetical protein that belongs to the Tryp_SPC (trypsin-like serine proteases) superfamily and is encoded by the gene AN2366.2. This analysis was carried by Dr Joe Gray in the Newcastle University Pinnacle facility for Biological MS.



Fig 5. 5: Mascot identity database searching analysis of PNM1B.

The MASCOT score for Q5BAR4_EMENI is 252; threshold score = 25; probability of a random match = 1 in 10^{22} . The MASCOT score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. The bar chart represents the significance of the recognised protein. The top scoring peptide matches for the spectrum are shown in red.

BLASTP2 Result



of [Bork Group's Advanced BLAST2 Search Service at EMBL](#)
[back to BLAST2](#)



BLASTP 2.OMP-WashU [16-Dec-1999] [irix6-r10k-L64 23:35:48 16-Dec-1999]

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Reference: 1. Gish, W. (1996-1999) <http://blast.wustl.edu>

2. Shevchenko A, Sunyaev S, Loboda A, Shevchenko A, Bork P, Ens W, Standing KC. (2001), Anal Chem 73(9),1917-26:
Charting the proteomes of organisms with unsequenced genomes
by MALDI-quadrupole time-of-flight mass spectrometry and
BLAST homology searching
[Abstract](#)

Query= query_sequence
(614 letters)

Database: nrdb-95%
2,078,555 sequences; 597,106,688 total letters.
Searching.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100% done

Color Key: red = positive hit; green = borderline hit; black = negative result

Summary:	Get the selected sequences	Reset
Sequences producing High-scoring Segment Pairs:	High Score	Total Score
./:tr emb l AACD01000039 AACD01000039_17 product: "hypotheti...	76	368 <input checked="" type="checkbox"/>
./:sptrembl Q7RTY6 Q7RTY6 Marapsin 2 precursor.//:sptrembl...	62	100 <input type="checkbox"/>
./:sptrembl Q64X76 Q64X76 Hypothetical protein.//:sptrembl...	67	67 <input type="checkbox"/>
./:tr emb l AACY01534220 AACY01534220_2 product: "unknown"; ...	61	61 <input type="checkbox"/>
./:sptrembl Q7MUN1 Q7MUN1 Hypothetical protein.//:sptrembl...	61	61 <input type="checkbox"/>
./:pironly S29485 S29485 GTP-binding protein o-ral - Pacif...	51	51 <input type="checkbox"/>
Get the selected sequences	Reset	

Query sequence and locally-aligned HSPs from database sequences

	0	130	260	390	520	614
query_sequence						
^ v trembl AACD01000039						
^ v sptrembl Q7RTY6 Q7		-		- - -	- - -	-
^ v sptrembl Q64X76 Q6				- -		
^ v trembl AACY0153422		-				
^ v sptrembl Q7MUN1 Q7					-	
^ v pironly S29485 S29			-			

Alignments:

^ = trembl|AACD01000039|AACD01000039_17 product: "hypothetical protein";
Aspergillus nidulans FGSC A4 chromosome VII ANcontigl.39, whole
genome shotgun sequence.
Length = 249

Total Score: 368

	0	50	100	150	200	249
trembl AACD01000039 A						
Local hits (HSPs)		- - -		-		-
Score = 76 (39.4 bits)						
Identities = 10/12 (83%), Positives = 10/12 (83%)						

Fig 5. 6: BLAST homology searching analysis of PNM1B.

The significant match is Q5BAR4_EMENI, score = 368. The top scoring protein match for the spectrum is shown in red.

BLAST searches of non-redundant protein databases revealed that Q5BAR4 is most closely similar to serine proteases in Diptera (45% identity and 67% similarity to *Anopheles gambiae*) but no matches to fungal homologues were identified in the top 100 hits. A more selective BLAST search of fungal genomes in the NCBI database revealed potential homologues in only four species *Alternaria brassicicola* (42% identity, 61% similarity), *Phaeosphaeria nodorum* (44% identity, 59% similarity), *Pyrenophora tritici-repentis* (42% identity, 55% similarity), *Botryotinia fuckeliana* (35% identity, 48% similarity) and *Trichoderma reesei* (34% identity, 47% similarity). There are also shorter sequence region matches, such as *Fusarium oxysporum* (55% identity, 65% similarity from amino acids 148-243), *Gibberella zeae* (53% identity, 64% similarity for from amino acids 148-243), and *Nectria haematococca* (50% identity, 64% similarity for from amino acids 148-243) for a 96 amino acids region. Only 3 *Aspergillus* species have potential homologues and the highest match is found in *Aspergillus flavus* (32% identity, 49% similarity). There appears to be no homologue in *Neurospora crassa*. This BLAST result shows that Q5BAR4 (PNM1B) is not universally conserved in fungi.

In summary, the most likely candidate protein for PNM1A is the subtilase Q5BGU2_EMENI encoded by the gene AN0238.2, and the most likely candidate protein for PNM1B is Q5BAR4_EMENI encoded by AN2366.2.

5.3 Benzamidine sensitivity of PNM1A and PNM1B

As PNM1B was predicted to be a trypsin-like protease, bovine trypsin was assayed in the NmrA-based proteolysis assay in parallel with purified PNM1B (protease active fractions from Superdex column). The amounts of 10 μ l of fractions containing PNM1B eluted from a Superdex column and 0.01 μ g bovine trypsin were incubated with 2 μ g NmrA at 25°C for 30 min. Inspection of Fig 5.7 shows that bovine trypsin generates similar proteolytic fragments to those produced by PNM1B thereby adding strength to the interpretation that PNM1B is a trypsin-like protease.

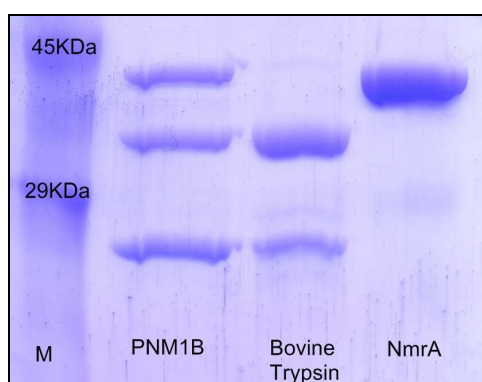


Fig 5. 7: NmrA proteolysis assay for PNM1B and bovine trypsin.

10 μ l of a single PNM1B containing fraction following DEAE FF and Superdex chromatography and 0.01 μ g bovine trypsin were incubated with 2 μ g purified NmrA at 25°C for 30 mins. Purified NmrA was used as a non-digested control. The positions of 29 kDa and 45 kDa markers are indicated.

The activity of serine proteases is typically inhibited by benzamidine, and as both PNM1A and PNM1B are predicted to be serine proteases, their sensitivity to benzamidine was tested. PNM1A and PNM1B were incubated at room temperature with 10 mM and 1 mM benzamidine, respectively, for 15 mins before incubation with 2 μ g NmrA. The final concentration of benzamidine in the PNM1A digest was 6.7 mM and for PNM1B was 0.67 mM. Samples lacking benzamidine were incubated in parallel as controls. The results in Fig 5.8 show that activity of PNM1B was completely inhibited by 0.67 mM benzamidine and that PNM1A was insensitive to the presence of 6.7 mM benzamidine. It is unusual for serine proteases to be insensitive to benzamidine; therefore this information and the difference in the estimated and predicted MW of PNM1A taken together do not permit an unambiguous assignment of the identity of PNM1A.

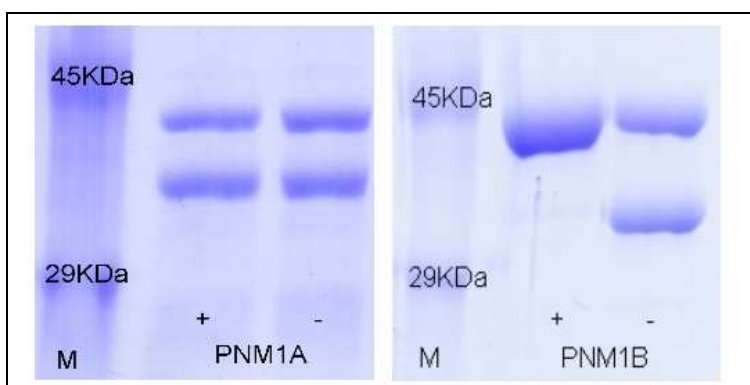


Fig 5. 8: Benzamidine sensitivity tests for PNM1A and PNM1B.

Benzamidine was pre-incubated at room temperature for 15 mins at a concentration of 10 mM with 10 μ l volumes of fractions containing PNM1A or at a concentration of 1 mM with 10 μ l volumes of fractions containing PNM1B purified by DEAE FF and Superdex chromatography. This was followed by incubation with 2 μ g NmrA at 25°C for 30 mins in a final volume of 15 μ l, giving a final concentration of benzamidine 6.7 mM for PNM1A and 0.67 mM for PNM1B. + = with benzamidine pre-incubation. - = no benzamidine pre-incubation. The positions of 29 kDa and 45 kDa markers are indicated.

5.4 The existence of a third protease-PNM1C

When crude cell free extracts were assayed in the NmrA-based proteolysis assay in the presence of 1 mM benzamidine (see Fig 5.9) the proteolytic product NM1B still appeared to be generated. This apparent production of NM1B could have been due to the benzamidine only partially inhibiting PNM1B or it could be that a third benzamidine-insensitive protease was producing a product indistinguishable by SDS-PAGE from NM1B.

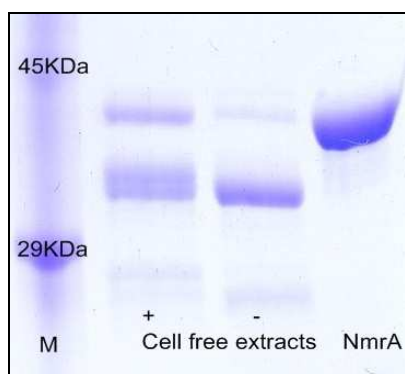


Fig 5. 9: NmrA proteolysis assay of strain R21 crude cell free extracts in the presence and absence of benzamidine.

Benzamidine was pre-incubated at room temperature for 15 minutes at a concentration of 1 mM with cell free extract. 1 μ g of these cell free extracts were then incubated with 2 μ g NmrA at 25°C for 30 mins at the same benzamidine concentration in a final volume of 10 μ l. + = with benzamidine. - = no benzamidine. Purified NmrA was used as a non-digested control. The positions of 29 kDa and 45 kDa markers are indicated.

To distinguish between these two possibilities, a benzamidine titration experiment was carried out. The amount of 1 μ g of cell-free extracts were pre-incubated with benzamidine in the concentration range 0.05 mM to 2 mM at room temperature for 15 minutes, followed by the incubation with 2 μ g NmrA at 25°C for 30 mins at the same benzamidine concentrations. A parallel sample lacking benzamidine was used as a control for comparison. The results presented in Fig 5.10 show that 0.5 mM benzamidine is sufficient to inhibit PNM1B activity and that there is no reduction in protease activity at concentrations of benzamidine of 0.1 mM or less. These observations support the interpretation that *A. nidulans* produces a third, benzamidine insensitive protease activity in response to nitrogen starvation. On the basis of these observations a minimum of 0.5 mM benzamidine was added to protease assays when the inhibition of PNM1B activity was required.

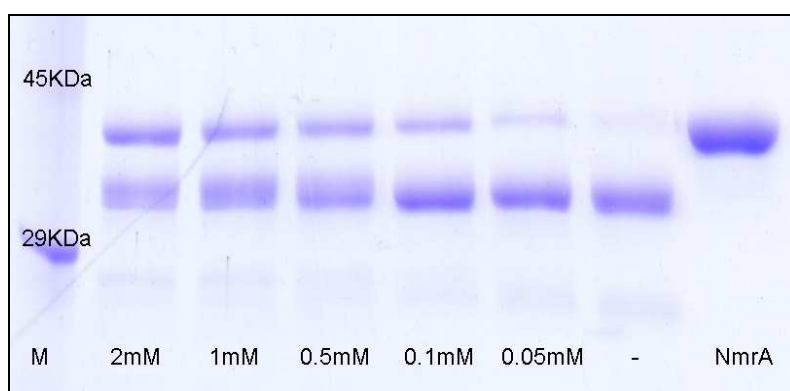


Fig 5. 10: NmrA proteolysis assay of strain R21 crude cell free extracts with different concentration of benzamidine.

Cell free extracts were pre-incubated with 0.05 mM, 0.1 mM, 0.5 mM, 1 mM and 2 mM benzamidine at room temperature for 15 mins. 1 μ g of these cell free extracts were then incubated with 2 μ g NmrA at 25°C for 30 mins at the same corresponding benzamidine concentration in a final volume of 10 μ l, respectively. A parallel incubation with no benzamidine was used as a control. - = no benzamidine present; + = benzamidine present. Purified NmrA was used as a non-digested control. The positions of 29 kDa and 45 kDa markers are indicated.

To further and more directly to prove the existence of a third protease, the elution profile generated from the chromatographic separation on a Hi Trap DEAE FF column of the proteins in a cell free extract (see section 5.1) was screened in the NmrA-based assay in the presence and absence of 1 mM benzamidine. When the two profiles are compared (see Fig 5.11), a third peak of protease activity, designated PNM1C, which is not benzamidine sensitive is evident. The elution of PNM1C overlaps the leading edge of the PNM1B peak. The previous analysis that determined the identity PNM1B used fractions from the central and trailing edge of the protease peak eluting from the Hi Trap DEAE FF column and were therefore free of PNM1C.

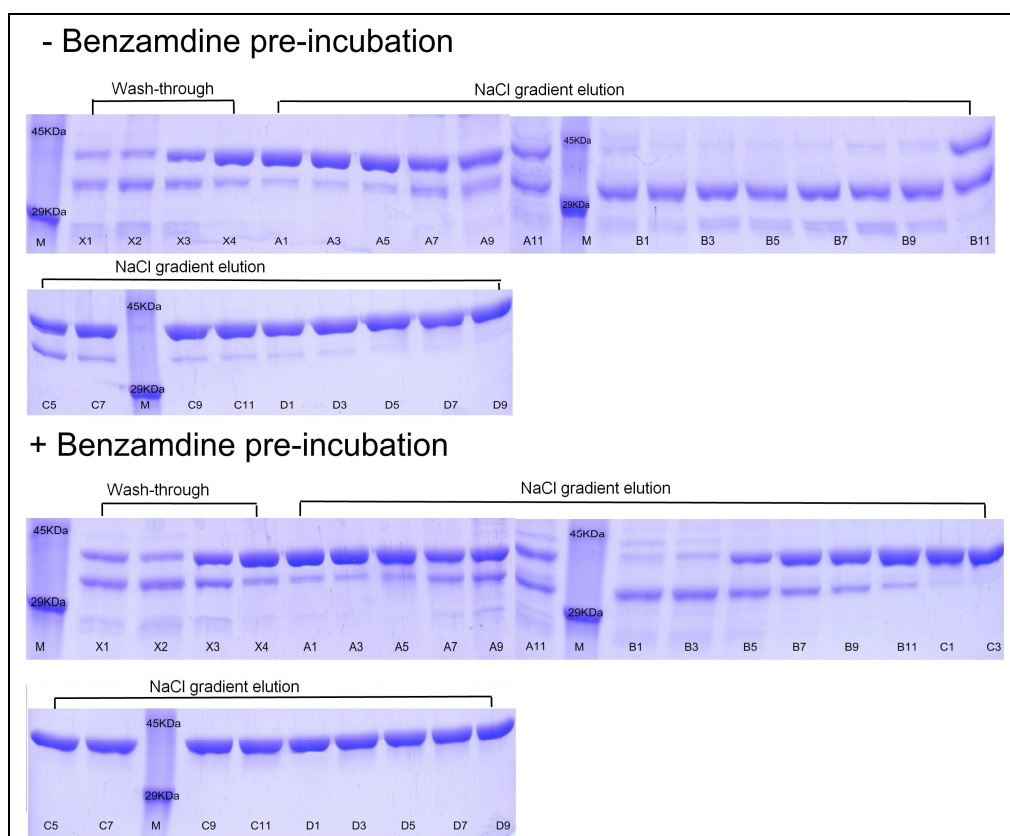


Fig 5. 11: Comparison of the proteolytic activities of wild type R21 in the presence and absence of 1 mM benzamidine after separation by chromatography on a 5 ml Hi Trap DEAE FF column.

1 ml volumes from individual fractions were made with 1 mM benzamidine and incubated at room temperature for 15 mins. 10 μ l of these benzamidine treated fractions and 10 μ l from equivalent benzamidine-free fractions were incubated with 2 μ g NmrA in a final volume of 15 μ l (final benzamidine concentration = 0.67 mM). Fractions X1-X4 are the 10 ml wash-through fractions. Fractions A1-D9 are the 2 ml fractions eluted by the 0.0-1.0 M NaCl gradient. The positions of 29 kDa and 45 kDa markers are indicated.

5.5 Production of PNM1C is undetectable in an *areA* deletion strain

PNM1A, PNM1B and PNM1C are produced in response to nitrogen limitation or starvation and NmrA has sites that are hypersensitive to these proteases - these observations raise the possibility that PNM1A, PNM1B, and PNM1C may be involved in the post-translational regulation of NmrA activity. As the transcription activating protein AreA is a known target for NmrA binding it was of interest to know if the production of any of the three proteases was under the control of AreA. To test this hypothesis the induction of PNM1A, PNM1B and PNM1C was investigated in a strain deleted for the *areA* gene. Crude cell free extracts of nitrogen starved mycelia from both wild type strain R21 and *areA* deletion strain NKRB3-2, were screened in the NmrA-based proteolysis assay in the presence and absence of benzamidine as shown in Fig 5.12. Digestion of NmrA by crude cell free extracts reveals the presence of all PNM1A, PNM1B and PNM1C and digestion in the presence of benzamidine reveals the presence of PNM1A and PNM1C. As PNM1C and PNM1B generate major digestion products of around 32 kDa that are indistinguishable by SDS-PAGE analysis, the presence of PNM1C can only be observed when the activity of PNM1B is inhibited by benzamidine. As the major product of PNM1A is a substrate for PNM1B, in crude cell free extracts that have a high activity of PNM1B the activity of PNM1A is masked. Inspection of Fig 5.12 shows that all three proteases could be detected in the wild type R21 strain but that no PNM1C activity could be detected in the *areA* deletion strain. These observations show that in order to produce detectable levels of PNM1C activity a wild-type *areA* gene is necessary.

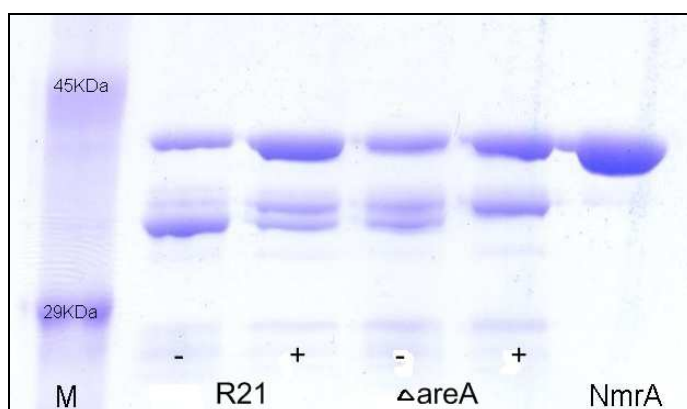


Fig 5. 12: NmrA proteolysis assay of crude extracts of wild-type R21 and *areA* deletion strain (NKRB3-2) in the presence and absence of benzamidine.

Benzamidine was pre-incubated at room temperature for 15 mins at a concentration of 1 mM with cell free extracts. 1 μ g of these cell free extracts and the untreated parallel ones were then incubated with 2 μ g NmrA at 25°C for 30 mins at the same benzamidine concentration in a final volume of 10 μ l, respectively. + = with benzamidine pre-incubation. - = no benzamidine pre-incubation. Purified NmrA was used as undigested control. The positions of 29 kDa and 45 kDa markers are indicated.

To test this interpretation more rigorously, crude cell free extracts derived from a nitrogen starved *areA* deletion strain (NKRB3-2) were chromatographically separated on a 5 ml Hi Trap DEAE FF column and eluted fractions screened in the NmrA-based proteolysis assay in the presence and absence of 1 mM benzamidine described in section 5.4 and the results of this analysis are shown in Fig 5.13. Inspection of Fig 5.13 shows that in the absence of benzamidine, two peaks, corresponding to PNM1A and PNM1B can be seen but that in the presence of benzamidine a single activity corresponding to PNM1A is apparent. These observations confirm that PNM1C is not detectable by the sensitive NmrA-based proteolysis assay and implies strongly that the production of PNM1C is under the control of AreA.

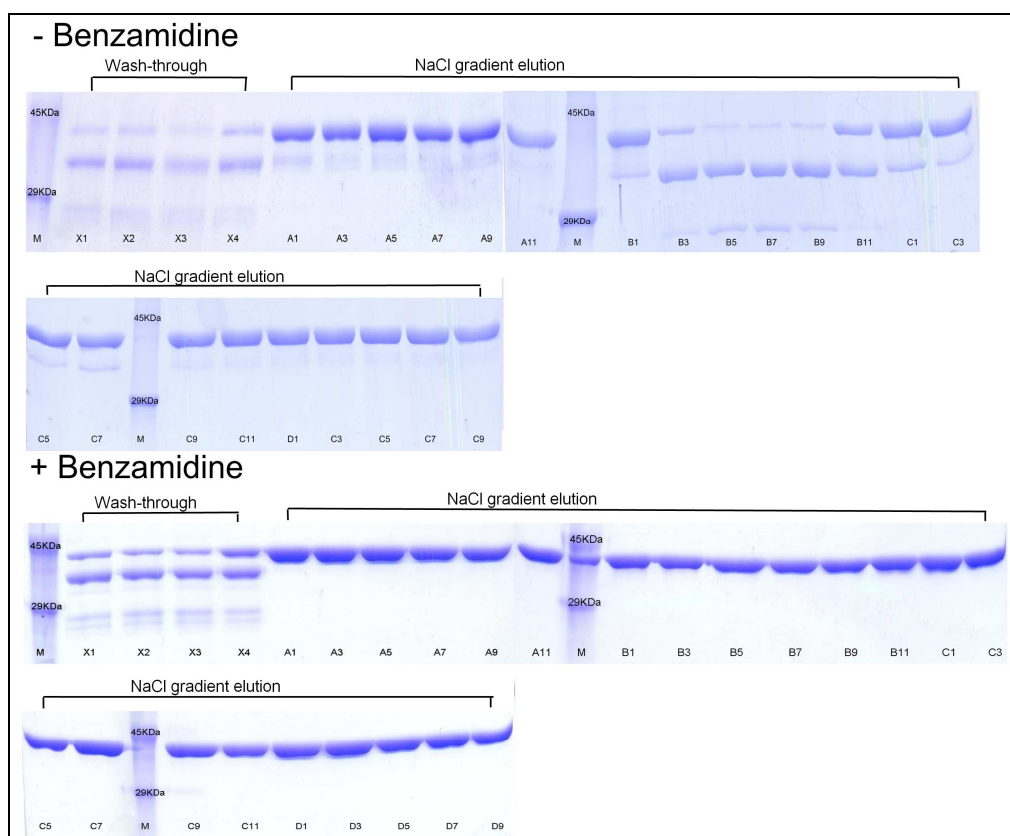


Fig 5. 13: Comparison of the proteolytic activities of *areA* deletion strain (NKRB3-2) in the presence and absence of 1 mM benzamidine after separation by chromatography on a 5 ml Hi Trap DEAE FF column.

1 ml volumes from individual fractions were made with 1mM benzamidine and incubated at room temperature for 15 mins. 10 μ l of these benzamidine treated fractions and 10 μ l from equivalent benzamidine-free fractions were incubated with 2 μ g NmrA in a final volume of 15 μ l (final benzamidine concentration = 0.67 mM). Fractions X1-X4 are the 10 ml wash through fractions. Fractions A1-D9 are the 2 ml fractions eluted by the 0.0-1.0 M NaCl gradient. The positions of 29 kDa and 45 kDa markers are indicated.

5.6 The induction profiles for the proteases PNM1A, PNM1B and PNM1C differ in their response to nitrogen starvation

In order to investigate the temporal induction of PNM1A, PNM1B and PNM1C in response to nitrogen starvation, wild type R21 spores were grown at 37°C for 19 hours in liquid minimal medium under nitrogen sufficient conditions (0.4% w/v glucose, 40 mM ammonium). Following a wash with nitrogen-free minimal media, the mycelia were transferred into new media lacking nitrogen source and incubated for further time periods of 0, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 16 hours and 24 hours before harvesting. Cell free extracts were prepared from the harvested mycelia and screened by the NmrA-based proteolysis assay in the presence and absence of 1 mM benzamidine. The results of this analysis are shown in Fig 5.14, where it can be seen that PNM1B activity is present in cultures starved of nitrogen for 2 or more hours, whereas PNM1A and PNM1C are only apparent in cultures starved of nitrogen for 6 or more hours. Furthermore, the assay carried out in the presence of benzamidine shows that the amount of the products of digestion by PNM1A and PNM1C remain relatively constant from 6 hours starvation onwards. These observations imply that when PNM1A and PNM1C are produced their concentration quickly reaches a plateau. However, the virtual disappearance of NmrA in general in assays lacking benzamidine implies that the concentration of PNM1B continues to rise over the 24-hour starvation period.

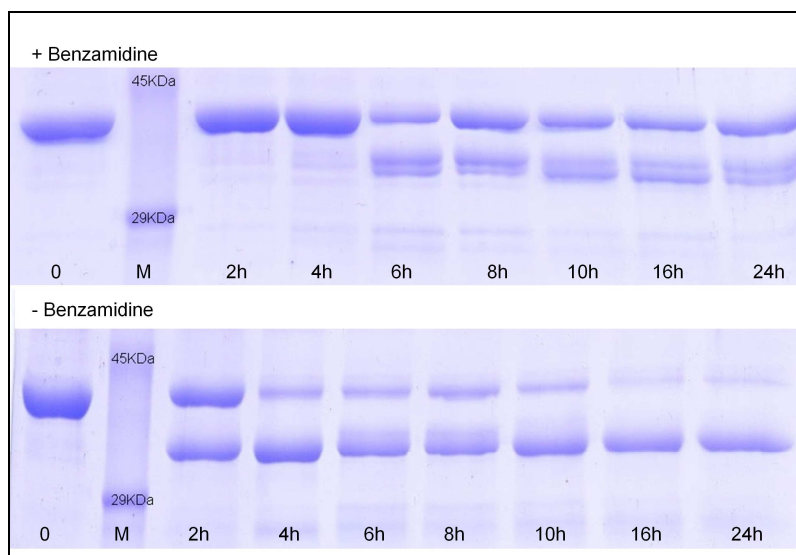


Fig 5. 14: NmrA-based proteolysis assay of cell free extracts prepared from wild-type R21 mycelium starved of nitrogen for various times in the presence and the absence of benzamidine.

Mycelia were grown at 37°C for 19 hours in liquid minimal medium containing 0.4% (w/v) glucose and 40 mM ammonium before washing in nitrogen-free minimal medium and transfer into new nitrogen-free and incubation for a further 0, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, 16 hours and 24 hours. Benzamidine was pre-incubated at room temperature for 15 mins at a concentration of 1 mM with cell free extracts. 1 µg of these cell free extracts and the untreated parallel ones were then incubated with 2 µg NmrA at 25°C for 30 mins at the same benzamidine concentration in a final volume of 10 µl, respectively. The positions of 29 kDa and 45 kDa markers are indicated.

It is important to note that when proteases PNM1A, PNM1B and PNM1C were isolated in bulk for study, they were purified from mycelium that has been starved of nitrogen for 4 hours. Given the observation described above, it may seem unusual that all three enzymes were present in mycelium after incubation in nitrogen-free medium for only 4 hours. The explanation for this apparent anomaly is that mycelia grown for purification of all 3 enzymes was grown for 18-19 hours in medium containing 10 mM ammonium compared to the 40 mM ammonium used in the experiments reported in this section. Growth for 18-19 hours in 10 mM ammonium will deplete the nitrogen source to a level that initiates production of the proteases before the mycelium is transferred to nitrogen free medium. Growth in 40 mM ammonium for 18-19 hours does not lower the concentration of the nitrogen source to a level that initiates protease production prior to the switch point to nitrogen-free medium as there are no protease activities at this point.

5.7 Synthesis of a gene encoding the protein Q5BAR4 (PNM1B)

PNM1B had been identified as protein Q5BAR4_EMENI, encoded by gene AN2366.2. Based on the published data in the Protein data bank (UniProtKB/ TrEMBL database), the complete open reading frame (ORF) sequence of AN2366.2 was synthesised by the company Blue Heron. This nucleotide sequence was optimised for expression in *E. coli* and was subcloned into the *E. coli* expression vector pET3a via 5'-NdeI and 3'-BamHI sites to yield the plasmid pMUT248. The complete synthesised sequence and corresponding protein sequence are shown in Fig 5.15.

Gene sequence (AN2366.2)

Nde 1

catatgaaaacggcagttaaactacagcccttcttagcttactctcgacagcaatggccgataaagcaattgtgggtggcgac
gacgcggaaattactgaatatccatatcaaattgctctgttatctggcgggctccctgatttggcgggtctattattagtagtaaat
atgtcgtaactgcggtcattgtacagacggcgctagcgctagctcattatccatccgtgcaggttctacttatcatgataaaggc
ggcaccgtggtagatgttgaagccattaccgtacatccagaatataatgtaacaccgtagataatgatatttctattttggaactg
gcggaagaattacaatttggcgacggtattaaagcgattgatttgcgagttcatcctcattaccaagcgaaggaaccattggta
ctgcaactggatgggggtgctctcacagaaggtggtaacgtaagtccgaattacaatacgtcgaagtcagggtgtatcgaaat
cccaatgttcttcagactacagcggtttcaatgaaattacagcatcaatgttttgcgccggggaagaggaaggtggtaaagatg
gttgtaaggcgattcaggcgggtccatttcagcagacggcgtcttaattggtattacttcttggggtaacggctgtgcacgtgc
aggttatccgggtgtttattcatcgccggcctattttcgcgatttcattcaacaagttacaggtttataa**BamH1**
ggatcc

Protein sequence (Q5BAR4_EMENI)

MKTAVKTTALLSLLSTAMADKAIVGGDDAEITEYPYQIALLSGGS LICGGSIISS
KYVV TAGHCTDGASASSLSIRAGSTYHDKGGTVVDVEAITVHPEYNANTVDND
ISILELAEELQFGDGIKAIDL PSSSSLPSEGTIGTATGWGALTEGGNVSPNLQYVE
VPVVSKSQCSSDYSGFNEITASMF CAGEEEGGK DGCQGDSGGPFAADGVLIGIT
SWGNGCARAGYPGVYSSPAYFRDFIQQVTGL

Fig 5. 15: The synthesised sequence of AN2366.2 and protein sequence of Q5BAR4_EMENI.

However, many trypsin-like proteases are synthesised as inactive precursor zymogens that are cleaved during limited proteolysis to generate their active forms. To determine if Q5BAR4_EMENI was likely to be processed in a similar manner, its amino acid sequence was analysed by the program SignalP 3.0. The results of this analysis are shown in Fig 5.16 and show that there is a 99.7% probability that amino acids 1-19 form a signal sequence and that there is a maximum cleavage site probability of 92.9% between amino acids 19 and 20. Based on this analysis, a gene encoding an N-terminally deleted form of PNM1B was synthesised and subcloned into the pET3a to yield plasmid pMUT249. The nucleotide sequence of pMUT249 is identical to pMUT248 except that it lacks the 5' codons 2-19.



Fig 5. 16: Prediction of signal peptide cleavage site of Q5BAR4_EMENI by SignalP 3.0 Server.

Plasmids pMUT248 and pMUT249 were transformed into the *E.coli* expression strains BL21 DE3, BL21 AI, BL21 Codon⁺ and BL21 pLysS and screened for recombinant protein production as described in Methods. Typical data are shown in Fig 5.17 and demonstrate that substantial recombinant protein production was achieved for both full length and N-terminally truncated PNM1B. Recombinant protein solubility was tested in three buffers as described in Methods and typical data are shown in Fig 5.18. Inspection of Fig 5.18 shows under all conditions tested the full length and N-terminally truncated PNM1B recombinant proteins were insoluble.

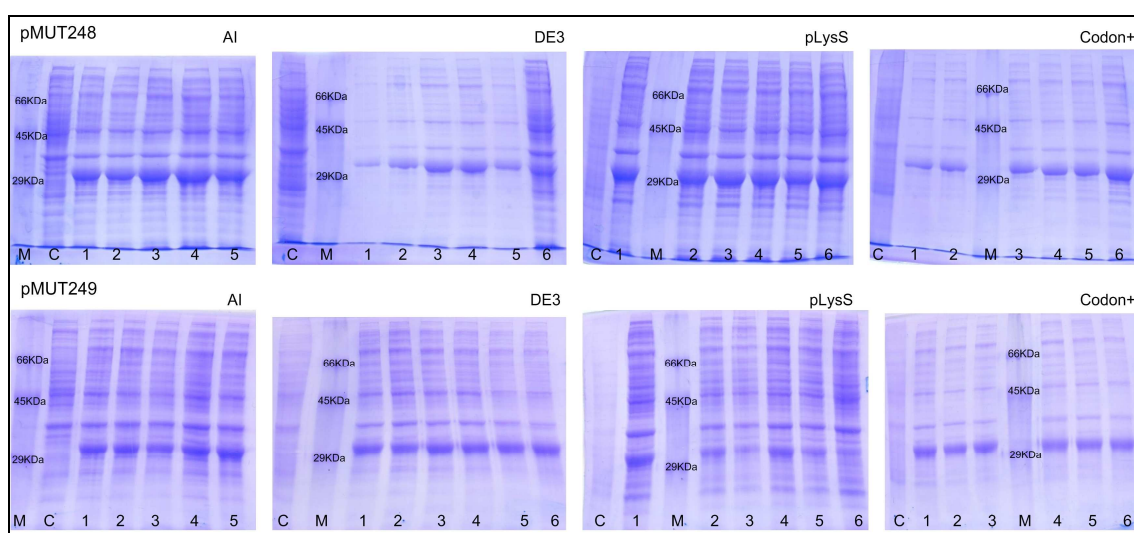


Fig 5. 17: Recombinant protein production of pMUT248/ pMUT249 in *E. coli* expression strains BL21DE3, BL21 AI, BL21 Codon⁺ and BL21 pLysS.

Transformant colonies were grown in 2ml liquid LB media (100 $\mu\text{g ml}^{-1}$ Ampicillin for all strains, 35 $\mu\text{g ml}^{-1}$ Chloramphenicol for pLysS and Codon⁺ strains only) at 30°C for 7 hours before 5-hour protein induction by adding 0.2 mg ml^{-1} IPTG for DE3, pLysS, Codon⁺ strains and 0.04% (w/v) arabinose for AI strain. For each expression strain, 5-6 transformant colonies were chosen screening for recombinant protein production as described in Methods. Boiled samples were run on 10% SDS-PAGE gels. Non-transformed expression colonies were used as negative controls (C). Positions of 66 kDa, 45 kDa and 29 kDa markers are indicated.

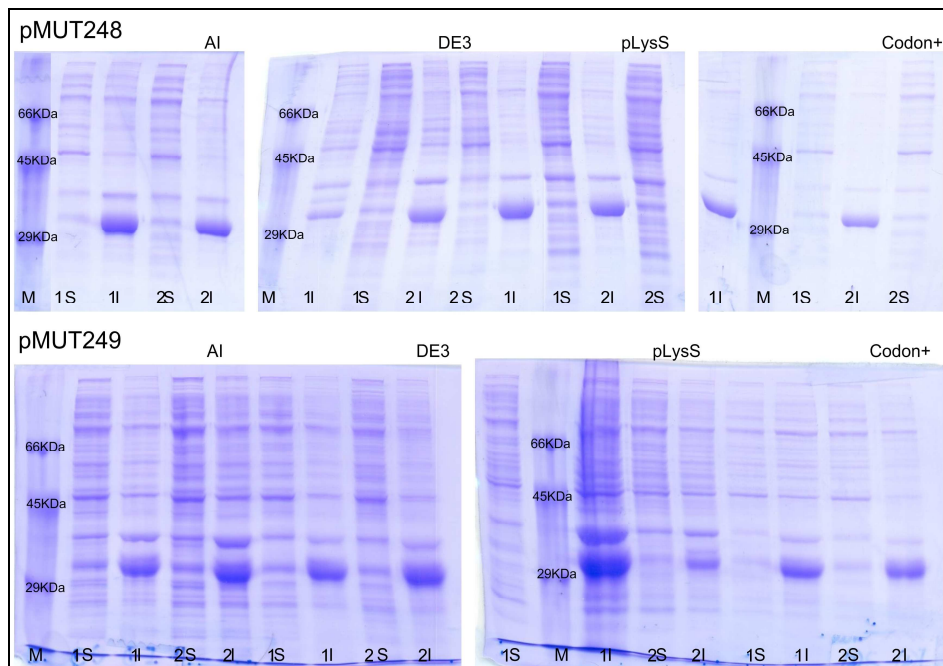


Fig 5. 18: Solubility test for recombinant proteins of pMUT248/ pMUT249 in *E.coli* expression strains BL21DE3, BL21 AI, BL21 Codon⁺ and BL21 pLysS.

For each expression strain, 2 recombinant protein productive transformant colonies were chosen for checking the protein solubility (see Methods). Cell pellets from each strain were resuspended and sonicated into two buffers: ① 0.1 M Tris-Cl, pH 7.5, 1 mM DTT buffer; ② 50 mM potassium phosphate, pH 7.2, 1 mM DTT buffer. After sonication, the supernatant from the centrifugation represents soluble portion while the cell pellet represents insoluble portion. S: soluble portion. I: insoluble portion. Positions of 66 kDa, 45 kDa and 29 kDa markers are indicated.

To try and obviate this problem, it was decided to try and solubilise the insoluble protein in buffer containing 6.0 M urea and to attempt refolding by slow removal of the urea by dialysis. Transformant cells BL21 DE3 with plasmid pMUT249 were used for the production of recombinant protein. After the urea-gradient protein refolding as described in Methods, the solubility of overproduced protein was analysed by SDS PAGE using a 10% acrylamide gel (see Fig 5.19). Inspection of Fig 5.19 shows that the most abundant protein was the N-terminally deleted PNM1B. 0.1 μ l and 0.5 μ l volumes of this solubilised protein were screened for proteolytic activity using NmrA as the substrate, but no protease activity could be detected.

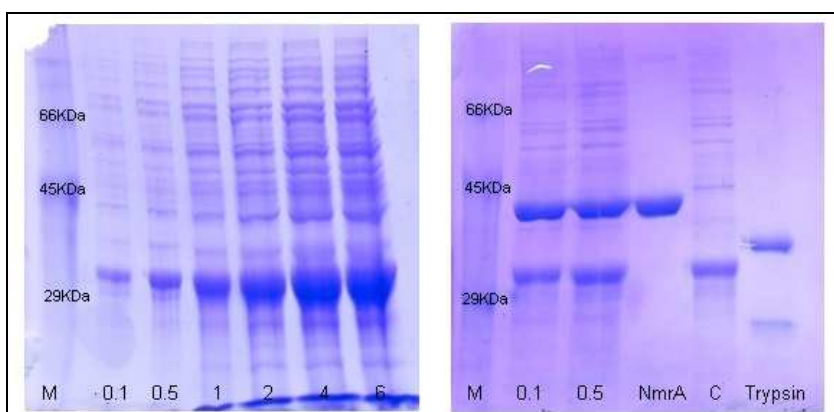


Fig 5. 19: Solubility and activity test of recombinant protein after urea refolding.

Left: increasing loadings (0.1 μ l, 0.5 μ l, 1 μ l, 2 μ l, 4 μ l, 6 μ l) of refolded recombinant protein electrophoresed on 10% SDS-PAGE gel. Right: NmrA proteolysis test for the refolded recombinant protein. 0.1 μ l and 0.5 μ l of solution were incubated with 2 μ g NmrA at 25°C for 30 mins. NmrA was used as non-digested control. C: 0.1 μ l refolded protein solution was used as background control. Trypsin: NmrA-based proteolysis of 0.01 μ g bovine trypsin was used as positive control. NmrA: recombinant NmrA protein for undigested control. 0.1: Incubation products of 0.1 μ l refold solution with NmrA protein. 0.5: incubation products of 0.5 μ l refold solution with NmrA protein. Positions of 66 kDa, 45 kDa and 29 kDa markers are indicated.

The absence of protease activity could have been due to one of several reasons. First, the protein may have been mis-identified in LC-MS, however, the probability of a random match to the data described previously is 1 in 10^{22} making the likelihood of incorrect identification very remote. Second, the sequence reported in the database may be incorrect thereby leading to the synthesis of a mutant and inactive protein. The gene AN2366.2 was PCR amplified from the wild type strain R153 genomic DNA and sub cloned into the vector pCR4-TOPO (Invitrogen) using the manufacturers recommended conditions. The forward and reverse PCR primers used were of the sequence: 5'- gct ata taa gga gtc aat tct-3' and 5'- gct tgc ata tat tgg aac tgg-3', respectively and are derived from the promoter and 3'-non-translated region. The cloned sequence was subjected to DNA sequencing and the translated amino acid sequence matched that encoded by the synthesised sequence. These observations imply that the refolded protein had a wild type amino acid sequence. The third possibility is that the final pool of soluble protein may not have folded correctly and therefore remained proteolytically inactive. One possible cause of incorrect folding may be due to an inability to form required disulphide bridges. Bovine trypsin digests NmrA in a manner indistinguishable from PNM1B as judged by SDS-PAGE electrophoresis, and PNM1B show 39% amino acid identity with the bovine enzyme (see Fig 5.20). To fold into an enzymatically active form the bovine enzyme has to make six disulphide bridges, and inspection of Fig 5.20 shows that the cysteine residues necessary for this are conserved in the PNM1B sequence. It is likely therefore that the reason the refolded PNM1B is proteolytically inactive is because it lacks the necessary disulphide bridges.

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			Score	E
Sequences producing significant alignments:			(Bits)	Value
1cl 56525	sp P00760 TRY1_BOVIN Cationic trypsin OS=Bos taurus...	166	6e-46	
ALIGNMENTS				
>1cl 56525 sp P00760 TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3 Length=246				
Score = 166 bits (419), Expect = 6e-46, Method: Compositional matrix adjust. Identities = 99/250 (39%), Positives = 139/250 (55%), Gaps = 18/250 (7%)				
Query	5	VKTTALLSLLSTAMA-----DKAIVGGDDAEITEYPYQIALLSGGSLI	GGSIISKYVV	59
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Sbjct	116	AASLNSRVASISLPTSCA--SAGTQCLISGWGNTKSSGTSYPDVLKCLKAPILSDSS	KS	173
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		Y G +IT++MF AG EGGKD QGDSGGP G L GI SWG+G A+ PGVY+		
Sbjct	174	AYPG--QITSNMF AGYLEGGKDS QGDSGGPVVCSGKLQGIWSWGSG	AQKNKPGVYTK	231
Query	236	PAYFRDFIQQ	245	
		+ +I+Q		
Sbjct	232	VCNYVSWIKQ	241	

Fig 5. 20: Alignment of PNM1B with Bovine trypsin.

The amino acids show 39% identity and 55% similarity between PNM1B and Bovine trypsin. Red bars show all the conserved cysteine residues in both sequences.

As the *E. coli* Origami (Novagen) expression strain can sometimes help correctly fold proteins that require disulphide bridges, Origami 2 and Origami 2 (DE3) competent cells were transformed with pMUT248 and pMUT249 and screened by 10% SDS-PAGE for soluble recombinant protein production. Typical results are shown in Fig 5.21 and Fig 5.22. Inspection of Fig 5.21 shows that in both cases there was substantial overproduction of recombinant protein in strain Origami 2 (DE3) but not in the strain Origami 2. Further solubility test shows that there is no better solubility of recombinant proteins in origami cells than that in normal *E. coli* cells (BL21 DE3). The soluble portions from each colony had been tested for their proteolytic activities by NmrA proteolysis and the results still remained negative.

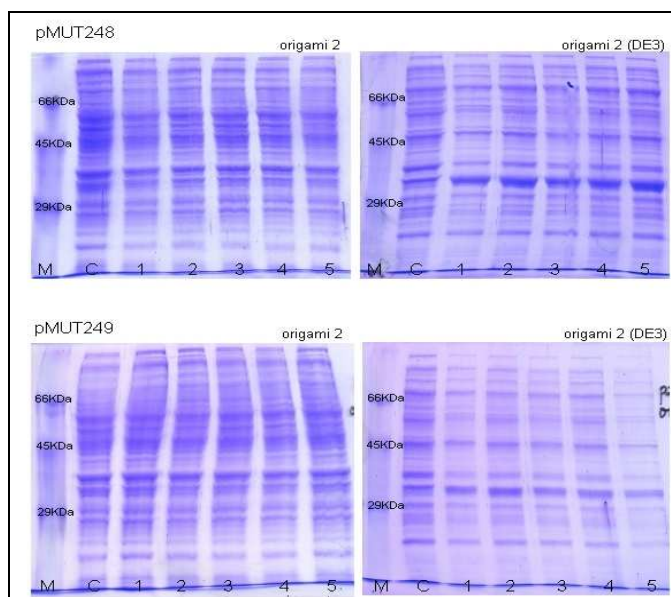


Fig 5. 21: Recombinant protein production by pMUT248 / pMUT249 in *E. coli* expression strains Origami 2 and Origami 2 (DE3).

Transformant colonies were grown in 2 ml liquid LB media ($100 \mu\text{g ml}^{-1}$ Ampicillin) at 30°C for 7 hours before 5-hour protein induction by adding 0.2 mg ml^{-1} IPTG. For each expression strain, 5-6 transformant colonies were chosen screening for recombinant protein production as described in Methods. Boiled samples were run on 10% SDS-PAGE gels. Non-transformed expression colonies were used as negative controls (C). Positions of 66 kDa, 45 kDa and 29 kDa markers are indicated.

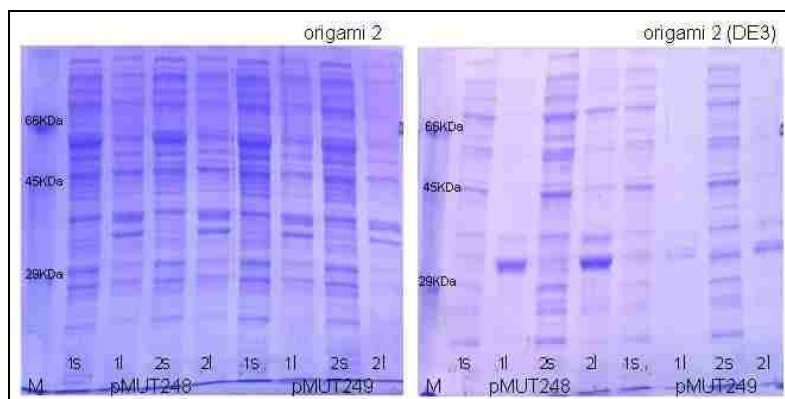


Fig 5. 22: Solubility test for recombinant proteins of pMUT248 / pMUT249 in *E. coli* expression strains Origami 2 and Origami 2 (DE3).

For each expression strain, two recombinant protein productive transformant colonies were chosen for checking the protein solubility (see Methods). Cell pellets from each strain were resuspended and sonicated into two buffers: ① 0.1M Tris-Cl, pH 7.5, 1mM DTT buffer; ② 50mM potassium phosphate, pH 7.2, 1mM DTT buffer. After sonication, the supernatant from the centrifugation represents soluble portion while the cell pellet represents insoluble portion. S: soluble portion. I: insoluble portion. Positions of 66 kDa, 45 kDa and 29 kDa markers are indicated.

Chapter 6 The effect of proteolysis on the biological activity of NmrA

6.1 NmrA undergoes ordered proteolysis

The data presented in Chapter 5 shows that *A.nidulans* produces three proteases in response to nitrogen starvation and that these proteases have hypersensitive sites for digestion within NmrA. These proteases are designated PNM1A, PNM1B and PNM1C and generate as their major digestion product-proteolytic fragments of around 32 kDa as judged by SDS-PAGE electrophoresis. Extended digestion with crude cell free extracts containing low concentrations of PNM1A, PNM1B and PNM1C generated a second major proteolytic fragment of MW approximately 25 kDa that was designated NM3. Similarly digestion of NmrA (approximately 2 hours) with higher concentration of purified PNM1A or PNM1B generated a fragment indistinguishable from NM3 in SDS-PAGE electrophoresis. Examples of this pattern of digestion are shown in Fig 6.1.

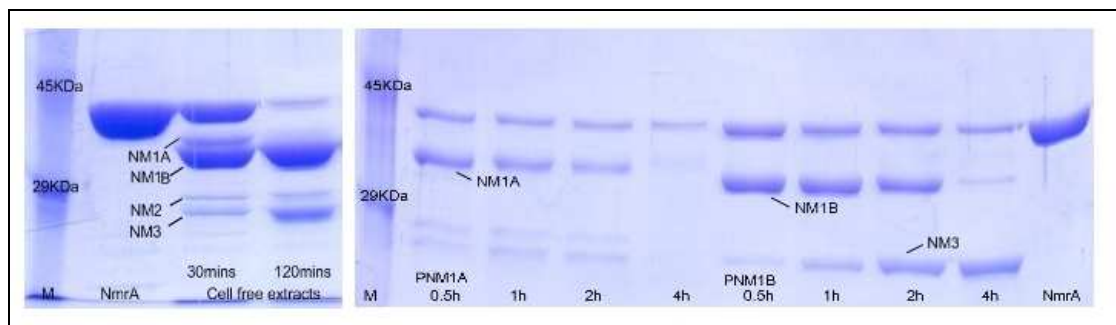


Fig 6. 1: Time course digestion of NmrA by crude cell free extracts and purified PNM1A and PNM1B.

1 μ g of crude cell free extracts (Left) and 10 μ l volumes of fractions containing PNM1A or PNM1B purified by Hi Trap DEAE FF and Superdex chromatography (Right) were incubated with 2 μ g purified NmrA at 25°C for different time lengths as indicated on the gels. The digestion products were separated by SDS-PAGE using a 10 % acrylamide separating gel and the positions of 45 kDa and 29 kDa markers are shown. The digestion products NM1A, NM1B, NM2, and NM3 are shown.

6.2 Mapping the sites of proteolytic cleavage in NmrA

The sites of proteolysis in NmrA for the production of the fragments NM1A and NM1B were determined by a combination of Edman sequencing and molecular weight (MW) determination using LC-MS (see Methods).

The N-terminal amino acid sequence of each NM1A and NM1B and that of the stock NmrA substrate was determined by Edman protein sequencing and in each case the sequence AQKKKTIA was recovered. These results demonstrated that (1) the recombinant NmrA stock lacked an N-terminal methionine and (2) that each of the digested NmrA fragments recovered started at the same N-terminus and so must have different C-termini. Combining this information with the MW determined by LC-MS, allows the cleavage sites generating NM1A, NM1B and NM3 to be identified.

Having determined that the stock of NmrA had no N-terminal methionine, its MW measured by MALDI-TOF is 38,663.8 Da which compares favourably with the predicted MW of 38,662.6 Da and shows that the recombinant protein was not modified in any other way. However, the MW of a sample of the same stock of NmrA that had been subjected to SDS-PAGE prior to LC-MS was found to be 38,737.5 Da. These measurements show that NmrA becomes covalently modified with an approximately 74 Da adduct (Table 6.1) as a result of the SDS-PAGE or the subsequent gel extraction prior to LC-MS.

Inspection of the LC-MS analysis of the proteolytic fragments (Fig 6.2 and Fig 6.3), NM1A and NM1B showed evidence of significant levels of oxidative microchemistry: every two neighbour peaks representing peptides that have 16 Da difference between

each other. The peptides with the lowest molecular weights were assumed to be the non-oxidative modified peptides. The measured molecular weights of NM1A and NM1B are 33,404.2 Da and 32,134.6 Da, respectively, as shown in Table 6.1. As a control, the MW of bovine carbonic anhydrase II was determined by this technique to be 29,023.7 Da. The measured value of the control MW showed Dalton level accuracy implying the same level of accuracy with the NmrA peptide MW determinations.

	Measured MW (mMW)	Predicted MW (pMW)	mMW- pMW	Cleavage site
NM1A	33,404.2 Da	33,330.2 Da (2-306)	74 Da	M306 and Q307
NM1B	32,134.6 Da	32,059.7 Da (2-291)	74.9 Da	K291 and G292
NM3	25252.9 Da	25178.15 Da (2-230)	74.8 Da	A230 and A231
NmrA from gel slice	38737.5 Da	38,662.6 Da	74.9 Da	
NmrA in solution	38,663.8 Da	38,662.6 Da	1.2 Da	

Table 6. 1: The measured and predicted MWs of NmrA and its proteolytic products.

The measured MWs of NmrA lacking the N-terminal methionine (NmrA 2-352) and the proteolytic fragment NM1B determined before and after SDS-PAGE electrophoresis and gel extraction are shown with the predicted MW derived from the amino acid sequence. A difference of 74.9 Da is evident in each case. Also shown are the measured MWs of the NmrA proteolytic products NM1A and NM1B after gel extraction along with the predicted MWs of C-terminally deleted NmrA possible candidate sequences.

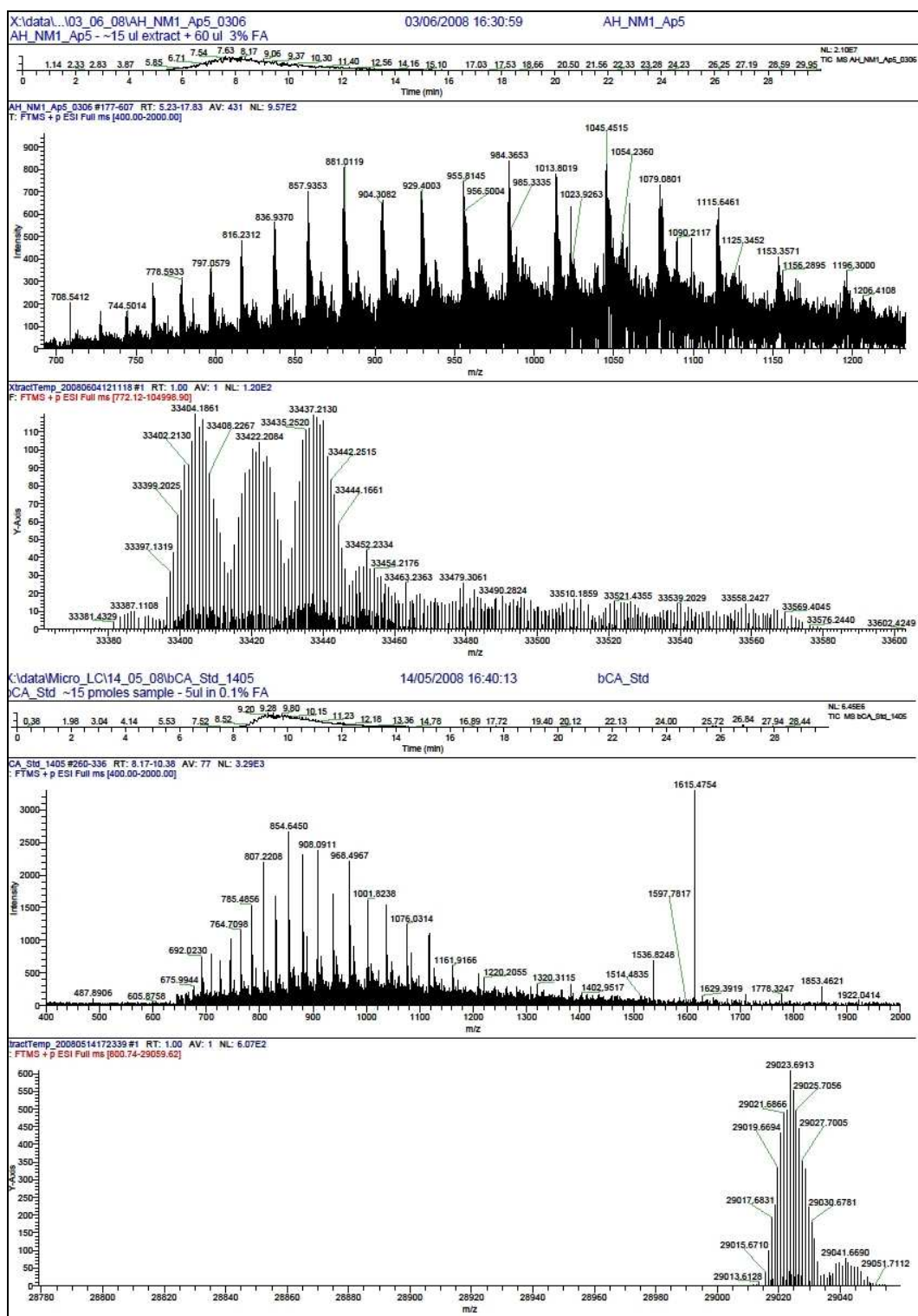


Fig 6. 2: Determining the MW of NM1A by LC-MS.

Upper: experimental peptide NM1A. The lowest MW is 33,404.1861 Da, considered as the measured MW of NM1A. Each neighbour peaks have 16 Da difference. Lower: control bovine carbonic anhydrase II (bCA). MW is 29,023.6913 Da.

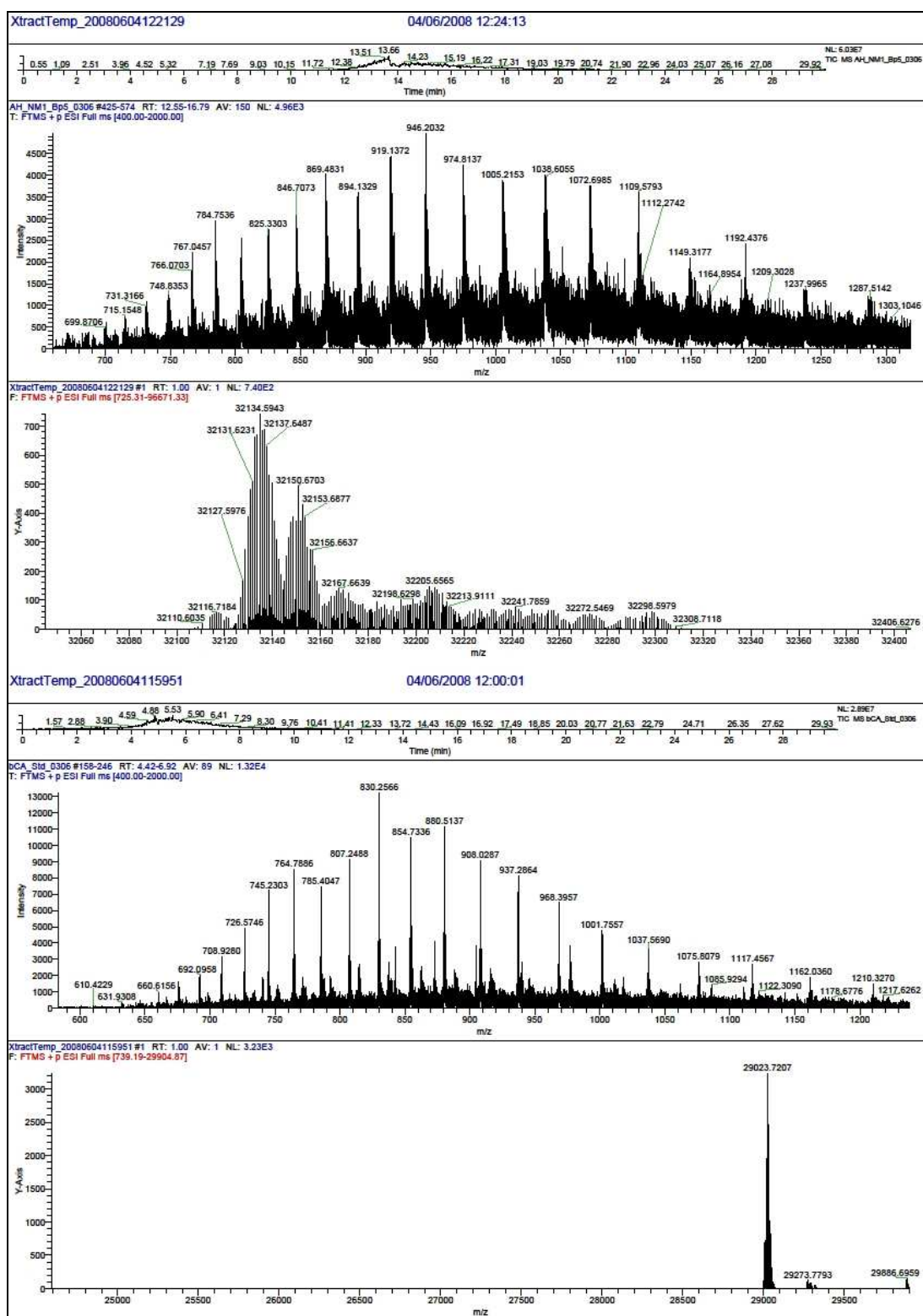


Fig 6. 3: Determining the MW of NM1B by LC-MS.

Upper: experimental peptide NM1B. The lowest MW is 32134.5943 Da, considered as the measured MW of NM1B. Each neighbour peaks have 16 Da difference. Lower: control bovine carbonic anhydrase II (bCA). MW is 29,023.7207 Da.

Knowing the N-terminus of the proteolytic products NM1A and NM1B and their accurate MWs allowed the deduction that the cleavage site of each protease lies within the C-terminal surface exposed non-ordered region of NmrA (residues 281-309) as shown in Fig 6.4. The LC-MS technique provides accuracy at the Dalton level; however there are no predicted cleavage products from NmrA that match the measured MW values with this level of accuracy. Given that the stock of NmrA is covalently modified as a result of SDS-PAGE and subsequent gel extraction for LC-MS, then providing digestion by PNM1A or PNM1B does not remove the site of modification, it is reasonable to infer that the proteolytic fragments NM1A and NM1B will be similarly modified. The peptides 2-291 and 2-306 differ from the measured MWs of NM1B and NM1A by +74.0 Da and +74.9 Da respectively, implying that they, like non-digested NmrA, have been modified with an approximately 74 Da adduct as a result of SDS-PAGE electrophoresis and gel extraction. In the case of the NM1B fragment this hypothesis was tested directly by repeating the MW determination with products of proteolysis that had not been subjected to SDS-PAGE electrophoresis. In this case the major digestion product had a measured MW of 32059.65 Da– a value that differs by 0.1 Da from the predicted MW of an NmrA fragment consisting of amino acids 2-291. Therefore the most likely cleavage site to generate peptide NM1A is between amino acids M306 and Q307, and to generate peptide NM1B is between K291 and G292.

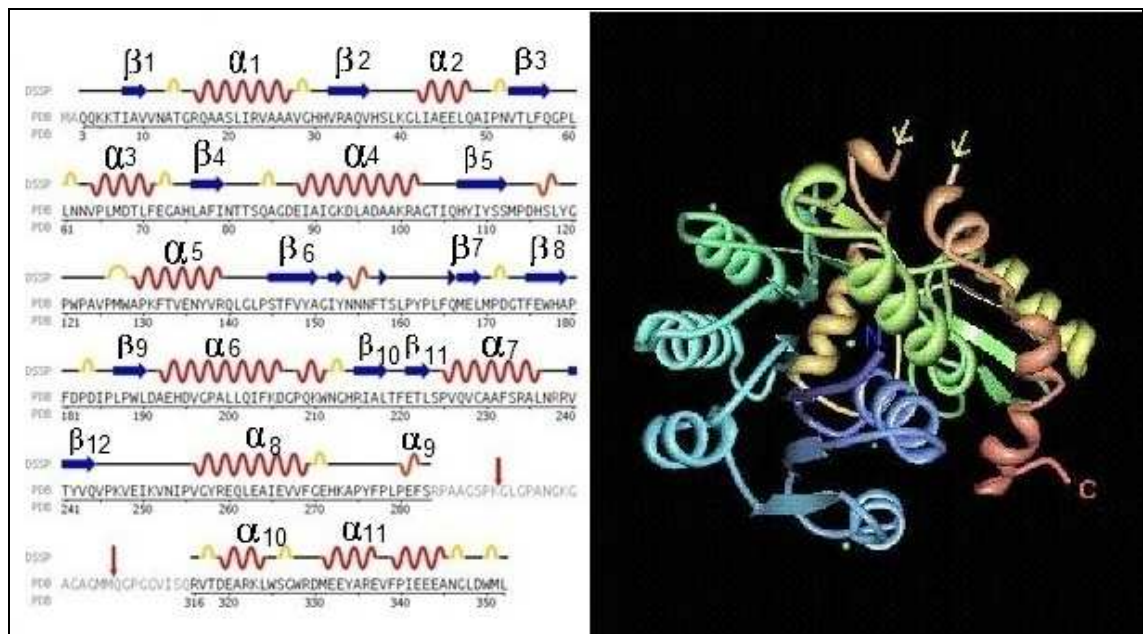


Fig 6. 4: Amino acid sequence and crystal structure of native NmrA protein (Monoclinic form). Left: the amino acid sequence of NmrA protein. Two red arrows indicates two digestion sites of PNM1A and PNM1B in the disordered loop region. Right: the crystal structure of NmrA protein. Two yellow arrows indicate the start and end points of the disordered surface loop region. The picture of crystal structure is from protein data bank (<http://www.rcsb.org/pdb/home/home.do>) by simple viewer.

The NM3 fragment of approximately 25 kDa formed by the digestion of NmrA with a crude cell-free extract of *A. nidulans* mycelium (therefore containing PNM1A, PNM1B and PNM1C activities) was gel purified and LC/MS experiments produced an estimated MW of 25,252.93 Da. Use of the Edman sequencing determined the N-terminal amino acid sequence as AQQKKTIA implying it was generated by cleavage within the surface exposed helix $\alpha 8$. The measured MW of this NmrA fragment differs by 3.7 Da from the predicted MW generated by digestion between residues A231 and F232. However, it is possible that this fragment is modified by the 74 Da adduct described above, and if 74 Da is subtracted from the measured MW the difference from the MW of a fragment generated by digestion between residues A230 and A231 is 1 Da. It is likely that this second proteolytic fragment is generated by digestion between A230 and A231. However, this site of digestion is incompatible with specificity of trypsin-like protease such as PNM1B and is therefore likely to be due to further digestion by PNM1A or PNM1C. Inspection of the amino acid sequence in this area shows that there is a potential PNM1B cleavage site (R234/A235) 4 amino acids C-terminal to A230. Thus in a mixture of the PNM1A, PNM1B and PNM1C, the NM3 product of PNM1B is a substrate for PNM1A and/or PNM1C.

All attempts to extract the PNM1B-generated fragment from SDS-PAGE electrophoresis at the same position as that of NM3 generated by cell free extracts were unsuccessful; consequently we were unable to determine the MW of this proteolytic fragment.

The *in vitro* generated data above show that NmrA is digested in an ordered fashion, with rapid cleavage occurring within the C-terminal unordered surface region

generating fragments NM1A and NM1B. NM1A is a substrate for the protease PNM1B therefore in crude cell-free extract NM1A is converted to NM1B. Similarly the NM3 fragment that is generated at a slower rate by PNM1B digestion within α -helix 8 is a substrate for PNM1A and PNM1C. The further digestion of NM3 by PNM1A, PNM1B and PNM1C does not generate stable intermediates implying that this fragment is disordered and that digestion is occurring at multiple sites simultaneously. Therefore, the proteolytic fragments NM1A and NM1B appear to be inherently resistant to proteolysis and require either an increase in digestion time or an increase in the concentration of protease of 10-fold or more to achieve quantitative conversion to lower MW fragments.

6.3 The inherent resistance of NM1A and NM1B to further proteolysis is enhanced by binding oxidised dinucleotides

NmrA is a member of a structural superfamily which includes the short-chain dehydrogenase/reductases. NmrA discriminates between oxidised and reduced dinucleotides, binding the former much more tightly than the latter (Lamb et al., 2003), however, the binding of oxidised dinucleotides has no effect on the ability of NmrA to bind to the AreA zinc finger and has no effect on the structure of NmrA. In order to identify possible biological consequences of dinucleotide binding, the resistance of NmrA to proteolysis by Superdex chromatographically purified PNM1A and PNM1B was tested in the presence and absence of NAD^+ , NADP^+ , NADH and NADPH over a 6-hour digestion. Fig 6.5 shows the results of typical experiments. Inspection of

Fig 6.5 reveals that, although all four dinucleotides have no obvious effect on the digestion of full length NmrA, the presence of oxidised dinucleotide NAD^+ enhances the inherent resistance of NM1A and NM1B to further proteolysis. In the presence of 0.5 mM NAD^+ , the digestion of NM1A and NM1B was dramatically reduced for up to 6 hours. This effect was also seen when NADP^+ was substituted for NAD^+ but was more variable and on average less pronounced. No protection was afforded by the addition of NAD (P) H. To test the hypothesis that it was the binding of oxidised dinucleotides to NmrA that was responsible for this effect the experiments were repeated with mutant NmrA that has a reduced ability to bind NAD^+ . The NmrA protein encoded by plasmid pMUT170 has the changes N12G/A18G (designated NmrA_{N12G/A18G}) and a 13-fold increase in the K_D for the binding of NAD^+ (Lamb et al., 2004). NmrA_{N12G/A18G} was purified in bulk and the digestion experiments repeated using Superdex-purified PNM1A and PNM11B – typical results are shown in Fig 6.6. Inspection of Fig 6.6 shows that the disappearance of NM1B from NmrA_{N12G/A18G} digested by PNM1B was not substantially modulated by the presence of NAD^+ . This observation supports the hypothesis that the protective effect of NAD^+ is through binding to NmrA and NM1B rather than by binding to and inhibiting the protease.

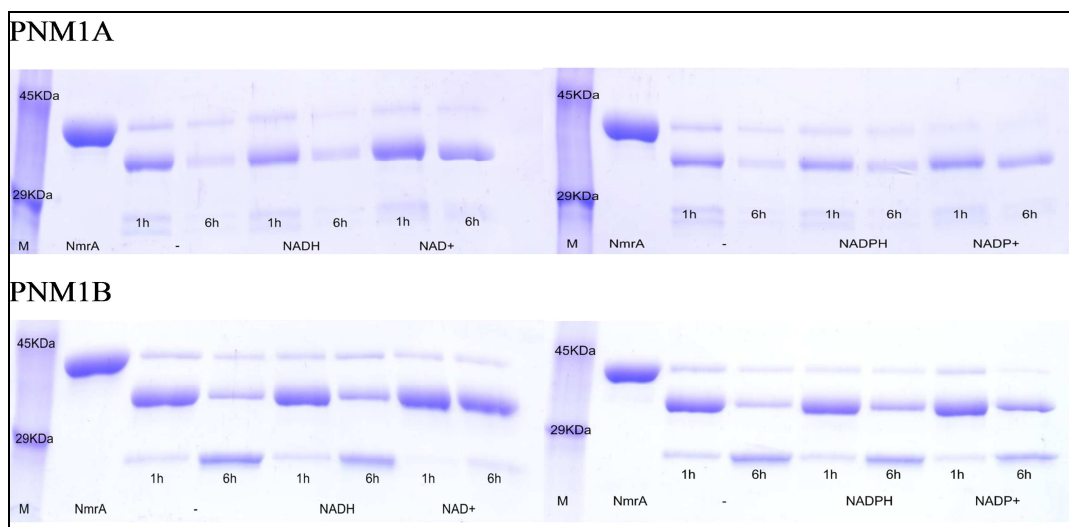


Fig 6. 5: Proteolysis of NmrA by PNM1A and PNM1B in the presence and absence of dinucleotides.

After Superdex chromatography 8 μ l fractions containing PNM1A activity and 5 μ l fractions containing PNM1B were assayed by NmrA-based proteolysis assay. The assay was carried out in the presence and absence of 0.5 mM NAD^+ , NADH, NADP^+ or NADPH. Assays were carried at 25°C and samples were taken at 1 hour and 6 hour intervals. NmrA protein was used as a non-digested control and the position of 29 kDa and 45 kDa markers are indicated.

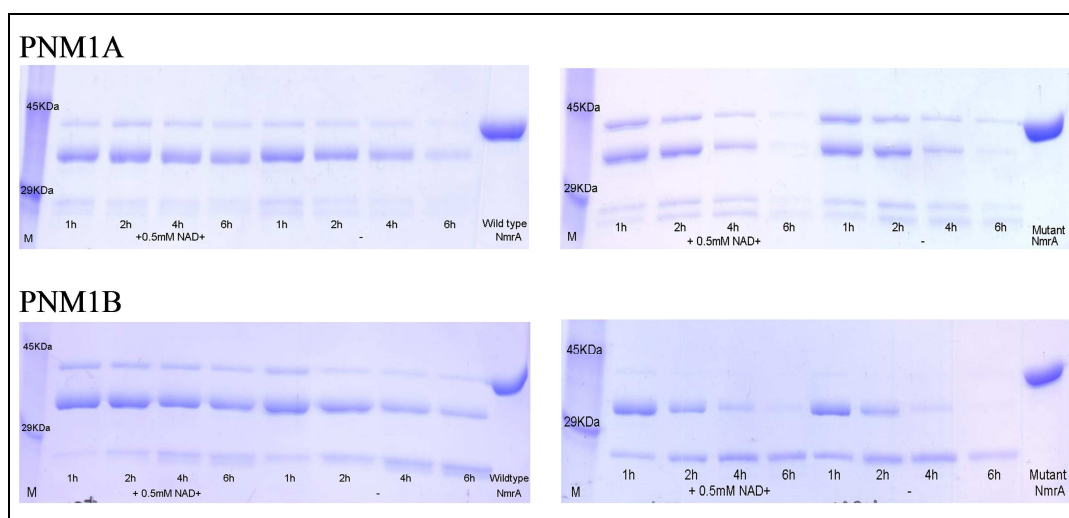


Fig 6. 6: Proteolysis of wild type NmrA (left) and N12G/ A18G mutant NmrA(right) by PNM1A and PNM1B at the presence/ absence of 0.5 mM NAD^+ .

After Superdex chromatography 8 μ l fractions containing PNM1A activity and 5 μ l fractions containing PNM1B were assayed by NmrA-based proteolysis assay. The assay was carried out in the presence and absence of 0.5 mM NAD^+ . Assays were carried at 25°C and samples were taken at 1 hour, 2 hour, 4 hour and 6 hour intervals. Wild type and mutant NmrA (NmrA_{N12G/A18G}) were used as a non-digested controls and the position of 29 kDa and 45 kDa markers are indicated.

As the primary proteolytic fragment remains stable during proteolysis in the presence of 0.5 mM NAD^+ and protease activity of PNM1B can be easily inhibited by adding benzamidine, the extent of proteolysis can be controlled manually. As described in Methods, the proteolytic products was fractionated by MONO Q column and monitored for the presence of NM1B by SDS-PAGE using 10% and 15% separating gels as shown in Fig 6.7. Inspection of Fig 6.7 shows that the proteolytic fragment NM1B co-purified with an approximately 6.6 kDa MW fragment. NM1B containing fractions were then pooled and dialysed into 50 mM potassium phosphate pH 7.2, 1 mM DTT to remove NaCl and NAD^+ prior to further analysis.

Mass spectrometry determined the accurate MW of the smaller fragment co-purified with NM1B as 6.625 kDa, a value identical (within experimental error) to that of the C-terminal fragment produced by the digestion of NmrA by PNM1B. Densitometry measurements using PROGENESIS software (Nonlinear Dynamics) showed that the NM1B and the C-terminal fragment of NmrA purified with a 1:1 stoichiometry. These observations indicate that when PNM1B cleaves NmrA within its C-terminal surface exposed loop the NM1B and the C-terminal fragment produced remain associated and that this association is not disrupted by MONO Q FPLC.

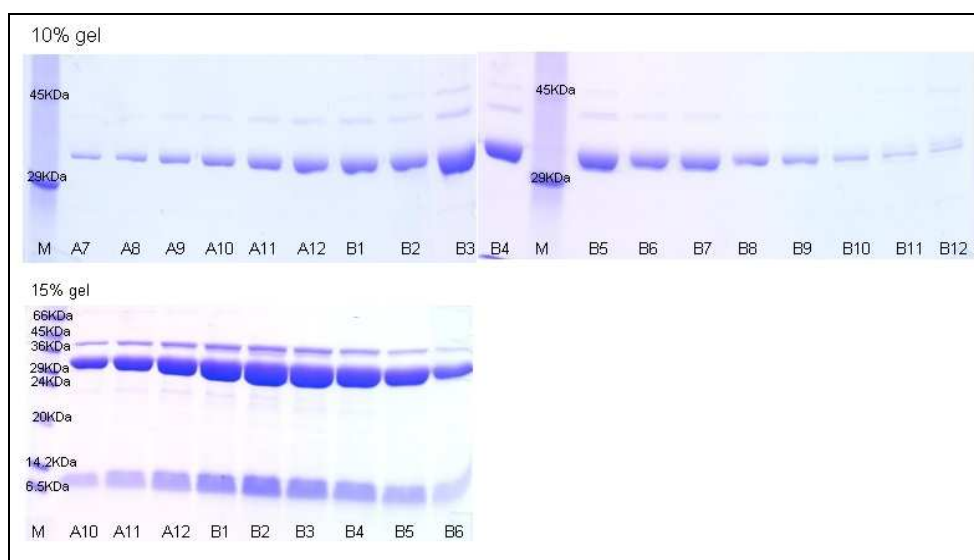


Fig 6. 7: SDS-PAGE analysis of PNM1B digested NmrA.

NmrA was digested by PNM1B in the presence of 0.5 mM NAD^+ and chromatographically purified on a MONO Q FPLC column. 5 μl of each fraction eluted by a 0-1.0 M NaCl gradient were analysed by SDS-PAGE using 10% and 15% separating gels. The positions of molecular weight markers are indicated.

The data shown in Fig 6.5 and Fig 6.6 imply that the NM1B is able to bind oxidised dinucleotides, and this interpretation was tested by independent microcalorimetric techniques. The mixture of NM1B and the C-terminal fragment of NmrA generated by digestion with PNM1B (designated NM1B+C term) was purified in bulk (see Methods) and analysed by Differential Scanning Calorimetry (DSC) in the presence and absence of 3 mM NAD⁺. Typical DSC results are shown in Fig 6.8, where it can be seen that the T_m of NM1B+C term is reduced compared to that of non-digested NmrA. However, in the presence of 3 mM NAD⁺ the T_m of NM1B+C term is increased relative to the value in the absence of dinucleotides. The isotherms are non-reversible, therefore they cannot be deconvoluted to derive binding constants, however they do demonstrate in a qualitative manner that NM1B+C term retains the ability to bind to oxidised dinucleotides.

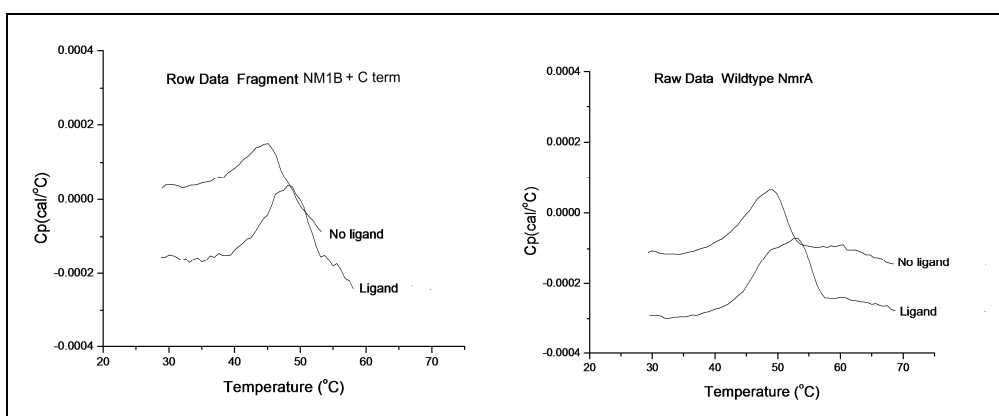


Fig 6. 8: DSC analysis of NAD⁺ binding to NmrA and NM1B+C term.

The concentration of NM1B+C-term and NmrA is 20 μM, the concentration of NAD⁺ is 3 mM and the experiment was carried out in 50 mM phosphate, pH 7.2, 1 mM DTT buffer. The scanning temperature range was from 25°C to 70°C at the rate of 90°C per hour.

NM1B+C term and, as a control, NmrA were also tested using Isothermal Titration Calorimetry (ITC) for the ability to bind to NAD^+ and typical results are shown in Table 6.2 and Fig 6.9. Table 6. 2 shows that the calculated 'c' value fell below 1 in the experiments with NM1B+C term, therefore the isotherms could not be accurately deconvoluted to derive binding constants. The reason for the low 'c' and stoichiometry values may be that the half life of the ligand binding properties of NM1B+C term is reduced. If, as is likely, this is the case then the necessary time taken (72 hours) to generate, purify and use NM1B+C term in ITC experiments means that the protein solution used was a mixture of active and inactive molecules. However, the ITC data do confirm directly that purified NM1B+C term retains the ability to bind NAD^+ and the value of the K_D (94 μM) for the binding of NAD^+ to NmrA is similar the previously published value (80 μM).

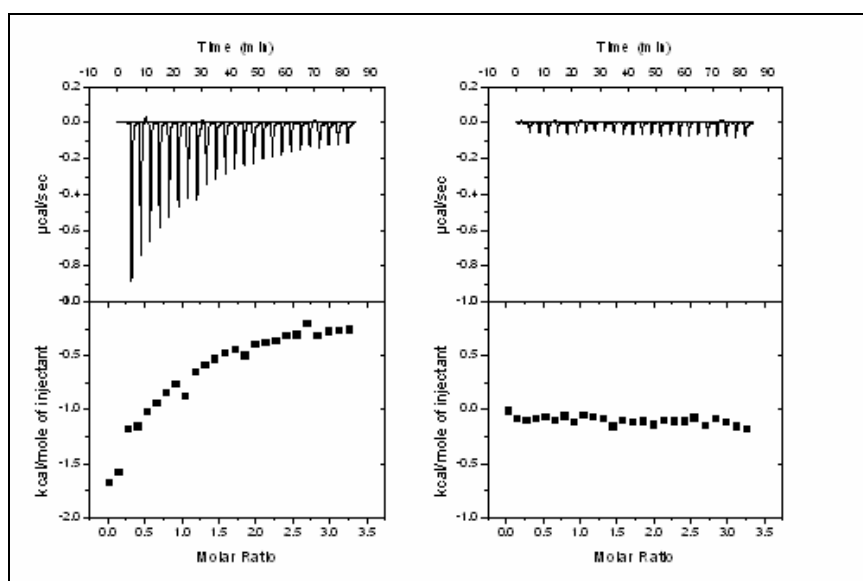


Fig 6. 9: ITC results of C-terminal fragment associated NM1B binding to NAD⁺.

The concentration of NM1B+C term is 112 μ M, the concentration of NAD⁺ is 2 mM and the reaction was carried out at 25 $^{\circ}$ C in 50 mM potassium phosphate pH 7.2, 1 mM DTT buffer. The ITC isotherm of NAD⁺ titrated into NM1B+C term (left) and NAD⁺ titrated into buffer alone (right) are shown for comparison..

Ligand in Cell	Ligand in syringe	n	K	K _D (μM)	Average K _D (μM)	ΔH (kcal mol ⁻¹)	ΔS (cal K ⁻¹ mol ⁻¹)	c
142 μM NmrA	2 mM NAD ⁺	0.7 (0.1)	1.06E4 (2.5E3)	94	94	3.4 (0.8)	6.98	1.0
112 μM NM1B+C term	2 mM NAD ⁺	0.6 (0.1)	9.50E3 (1.6E3)	105	102	4.4 (1.1)	3.65	0.6
112 μM NM1B+C term	2 mM NAD ⁺	0.6 (0.2)	9.93E3 (3.0E3)	100		3.7 (1.5)	6.03	0.6

Table 6. 2: Thermodynamic parameters for the binding of NM1B and NmrA to NAD⁺ using ITC analysis at 25 °C.

The binding was measured in 50 mM potassium phosphate pH 7.2, 1 mM DTT buffer by ITC. Shown are the values for n, the stoichiometry of binding; K_D, the apparent equilibrium dissociation constant; ΔH, the observed enthalpy; ΔS, the standard entropy change for single site binding. As the c values for NM1B+C term fall below 1 the K_D for the binding reaction cannot be reliably determined.

6.4 Proteolytic digestion within its C-terminal unordered loop abrogates the ability of NmrA to bind the AreA zinc finger.

The all data in section 6.4 imply that C-terminal fragment associated NM1B retains sufficient binding ability to NAD^+ , and we wished to know if the fragment mixture was able to bind to the AreA zinc finger. It is previously shown by ITC that NmrA binds endothermically to the C-terminal 213 amino acids of AreA (AreA₆₆₄₋₈₇₆) with a 1:1 stoichiometry and a K_D of approximately 0.26 μM (Lamb et al., 2003). Crystallographic studies and ITC analysis reveal that NmrA specifically interacts with the AreA Zf (residues 663-727) (Kotaka et al, 2008).

The interaction of NmrA and NM1B+C term with the AreA Zf was measured by ITC. The typical result is shown in Fig 6.10 while the thermodynamic parameters associated with the interaction are summarised in Table 6.3. In contrast to non-digested NmrA, when NM1B+C term was titrated onto AreA Zf, no significant heat exchanges over baseline controls were observed, implying no interaction. This observation can be rationalised by considering the crystal structure of NmrA in a complex with the AreA Zf. The AreA Zf interacts mainly through α -helices 1, 6 and 11 of NmrA in a combination of polar and hydrophobic contacts (Kotaka et al, 2008). Of the four hydrogen bonds between the AreA Zf and NmrA, three involve amino acids located within the C-terminal 27 amino acids of NmrA. Cleavage within the unordered surface loop will break the covalent link to the C-terminal peptide and is therefore likely to cause changes to its orientation relative to the NM1B fragment. Such changes could

disrupt the potential polar contacts of the proteolytic fragments mixture with AreA₆₆₃₋₇₂₇ and account for their observed inability to interact.

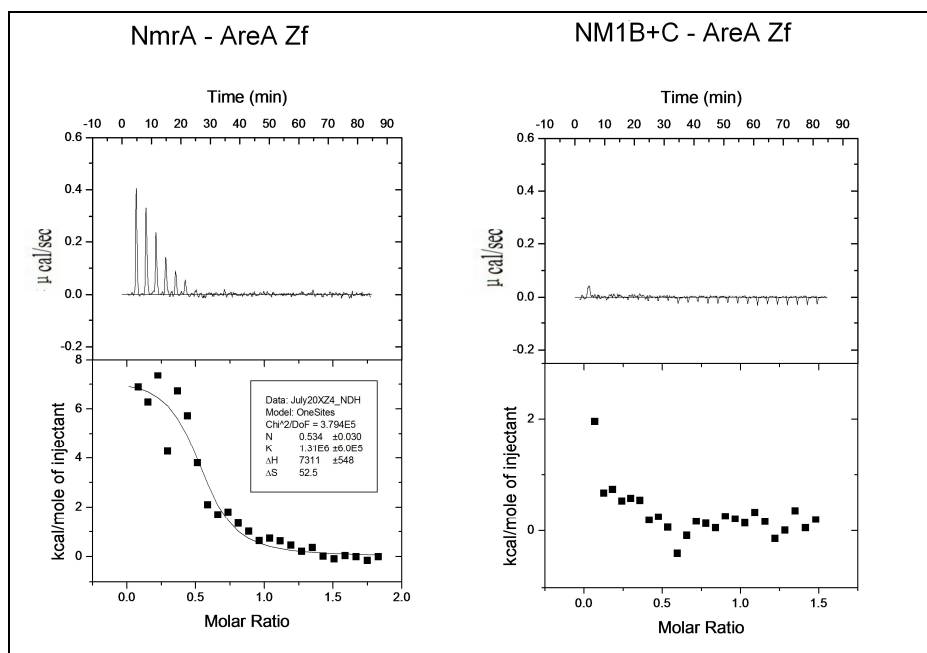


Fig 6. 10: ITC results of NmrA and C-terminal fragment associated NM1B binding to AreA₆₆₃₋₇₂₇.

The concentrations of NmrA and the C-terminal fragment associated NM1B are 247 μ M and 200 μ M, respectively. The concentration of AreA₆₆₃₋₇₂₇ was 25 μ M. The reaction temperature was 25°C.

Ligand in Cell	Ligand in syringe	N	K	K _D (μ M)	Δ H (kcal mol ⁻¹)	Δ S (cal K ⁻¹ mol ⁻¹)	<i>c</i>
25 μ M AreA Zf	247 μ M NmrA	0.527	1.18E6 (4.9E5)	0.84	7.931 (0.569)	54.4	15.5
25 μ M AreA Zf	247 μ M NmrA	0.534	1.31E6 (6.0e5)	0.76	7.311 (0.548)	52.5	17.5

Table 6. 3: Thermodynamic parameters for the binding of NmrA to NAD⁺ using ITC analysis at 25°C.

The binding was measured in 50 mM potassium phosphate pH 7.2, 1 mM DTT buffer by ITC. Shown are the values for n, the stoichiometry of binding; K_D, the apparent equilibrium dissociation constant; Δ H, the observed enthalpy; Δ S, the standard entropy change for single site binding.

To test the hypothesis that NmrA and NM1B+C term have some structural differences, their far-UV spectra were determined and compared (see Fig 6.11). Inspection of Fig 6.11 shows that there are small differences in the far UV spectra in the region 202-222 nm consistent with a subtle change in structure of NM1B+C term relative to NmrA. In ITC experiments NM1B+C-term bound NAD^+ (see Table 6.2 and Fig 6.9) however the calculated stoichiometry of the interaction (approximately 0.6) was lower than the value of 1 known from structural studies and the K_D could not be reliably calculated as the ' c ' value fell below 1 (Wiseman et al., 1989). The reason for the aberrant stoichiometry and low c value is possibly due to the subtle structural changes revealed by the far UV spectrum of the proteolytic fragment mixture. These changes may cause a reduction in the half life of its binding ability to NAD^+ . The necessary time taken (72 hours) to generate, purify and use of the C-terminal fragment associated NM1B in ITC experiments makes it likely that the protein solution used was a mixture of active and inactive molecules. However, the ITC data do confirm directly that the purified fragment retains the ability to bind NAD^+ .

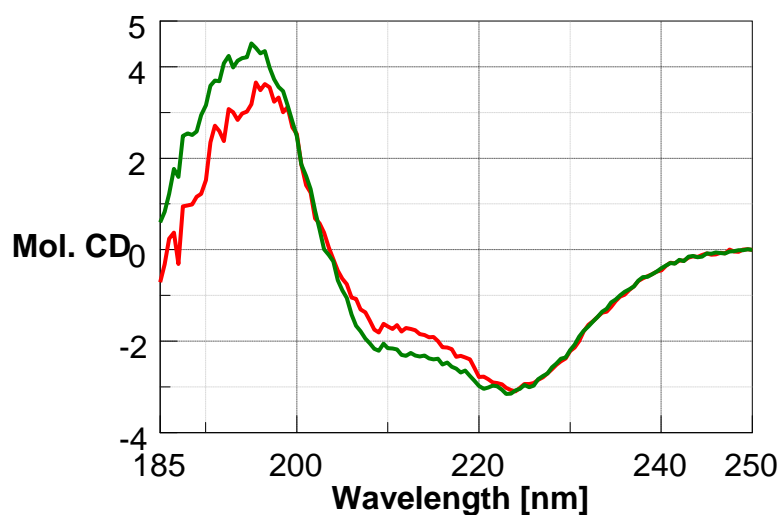


Fig 6. 11: Far UV circular dichroism analysis of NmrA and NM1B+C term.

The secondary structure of NmrA and NM1B+C term at a concentration of 0.51 mg ml^{-1} in 50 mM potassium phosphate pH 7.2, 1 mM DTT buffer was probed by far-UV CD spectroscopy. CD spectra for each protein were recorded from 10 accumulative scans of protein in buffer and buffer alone at 20°C using a Jasco J-810 spectropolarimeter. The buffer baseline was subtracted from the experimental value, the data adjusted for protein concentration and the results shown as molecular CD units. The trace with the greatest signal at 190 nm is from non-digested NmrA (green).

6.5 Synthesis of a gene encoding the fragment NM1B

In order to try and simplify the purification of NM1B without the C-terminal fragment a nucleotide sequence (optimised for expression in *E. coli*), encoding NmrA amino acids 1-191 was synthesised by the company Blue Heron. This nucleotide sequence was subcloned into the *E. coli* expression vector pET3a via 5'-NdeI and 3'-BamHI sites to yield the plasmid pMUT242. The complete synthesised sequence and corresponding protein sequence are shown in Fig 6.12.

Gene sequence
<p>Nde1</p> <p>catatgatggcacagcagaaaaaacattgccgttgtaatgcaaccggctcgcaggcagcaagcctgattcgtgttcagcagcagttggcatcatgttcgtgcacaggttcatagcctgaaaggctgattgcagaagagctgcaggcaattccgaatgttaccctgtttcagggtccgctgctgaataatgtccgctgatggataccctgtttgaagggtcacatctggcctttattaataccacctcaggctggatgaaattgccattggttaaagatctggcagatgcagaaaacgtgcaggcaccattcagcattatatttctagcatccggatcatagcctgtatggctcggcctgcagtccgatgtgggcaccgaaatcaccgtggaaaattatgttcgtcagctgggtctgccgagcacctctgtttatccgggtattataataataattttaccagcctgccgtatccgctgttcagatggaactgattccggatggcacctttgaatggcatgcaccgtttgatccggatattccgctgccgtggctggatgcagaacatgatgttggtccggcactgctgcagattttaaagatgtccgcagaaatggaatggatcatctattgcactgacctttgaaacctgtctccggtcaggtttgcagcatttagccgtgcactgaatcgtcgtgttacctatgttcagggtccgaaagtggagattaaagtgaatattccggtgggttatcgtgaacagctggaagcaatgaagttgtgttggcgaacataaagcaccgtattttccactgccggaatttagccgtccggcagccggtagcccgaataa</p> <p>BamH1</p> <p>gatcc</p>
Protein sequence
<p>MAQQKKTIAVVNATGRQAASLIRVAAAVGHHVRAQVHSLKGLIAEELQAIPNV</p> <p>TLFQGPLLNNVPLMDTLFEGAHLAFINTTSQAGDEIAIGKDLADA AKRAGTIQH</p> <p>YIYSSMPDHSLYGPWPAVPMWAPKFTVENYVRQLGLPSTFVYAGIYNNNFTSL</p> <p>PYPLFQMEMLPDGTFEWHAPFDPDIPLWLDAEHDVGPALLQIFKDGPKQKWN</p> <p>GHRIALTFTETLSPVQVCAAFSRALNRRYTYVQVPKVEIKVNIPVGYRE</p> <p>QLEAIEVVFGHEHKAPYFPLPEFSRPAAGSPK</p>

Fig 6. 12: The gene sequence and encoded amino acid sequence of fragment NM1B (1-191 amino acids of NmrA).

Plasmid pMUT242 was transformed into the *E.coli* expression strains BL21 DE3, BL21 AI, BL21 Codon⁺ and BL21 pLysS and screened for recombinant protein production as described in Methods. As shown in Fig 6.13, substantial recombinant protein production was achieved in strains BL21 AI and BL21 pLysS for the protein fragment NM1B both at 25°C and 30°C with less production in strain BL21 Codon⁺. The solubility of the recombinant protein produced in each *E.coli* expression strains was then tested in solubility Buffer 3 and Buffer 4 (see Fig 6. 13). Inspection of Fig 6.13 shows that the recombinant protein is highly insoluble in each strain in both buffers. However the data also implied that a small fraction of the recombinant protein may be soluble in buffer 1 in strain BL21 pLysS grown and induced at 25°C (see Fig 6.14). Based on both the recombinant protein production investigation and solubility tests, plasmid pMUT 242 was transformed into *E. coli* expression strain BL21 pLysS in preparation for bulk purification under the 25°C/15°C growth and induction regime. However when 50 g of cells were sonicated in buffer 1 and the clarified supernatant chromatographed on a Q sepharose column no recombinant NM1B protein could be detected. Given these observations work on purifying recombinant NM1B protein was discontinued. The reason the NM1B fragment of NmrA was insoluble may be because the C-terminal fragment is essential for NM1B to fold correctly. Alternatively the NM1B fragment may fold correctly but be highly unstable.

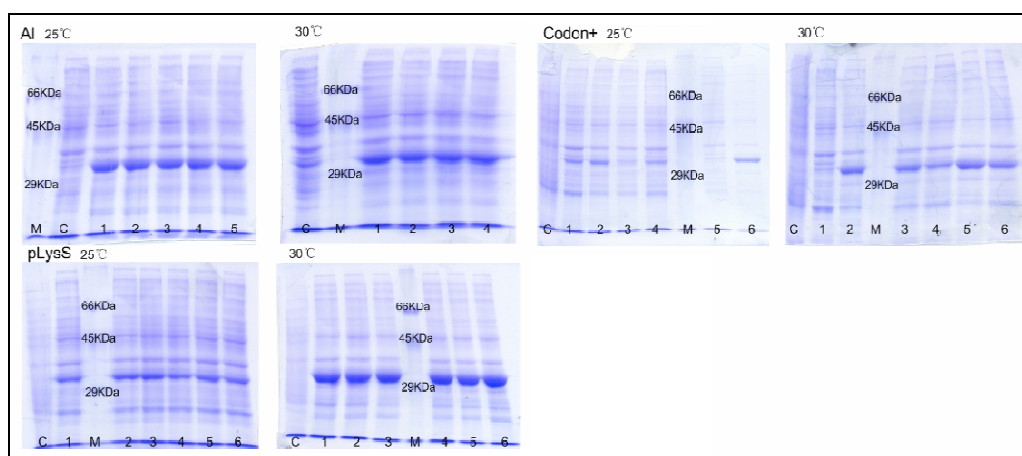


Fig 6. 13: Recombinant protein production of pMUT242 in *E. coli* expression strains BL21 AI, BL21 Codon⁺ and BL21 pLysS.

Transformant colonies were grown in 2 ml liquid LB media (100 $\mu\text{g ml}^{-1}$ Ampicillin for all strains, 35 $\mu\text{g ml}^{-1}$ Chloramphenicol for pLysS and Codon⁺ strains only) at 30°C for 7 hours or at 25°C for 10 hours before 5-hour protein induction by adding 0.2 mg ml^{-1} IPTG for pLysS, Codon⁺ strains and 0.04% (w/v) arabinose for AI strain. For each expression strain, 4-6 transformant colonies were chosen screening for recombinant protein production as described in Methods. Boiled samples were run on 10% SDS-PAGE gels. Non-transformed expression colonies were used as negative controls (C). Positions of 66 kDa, 45 kDa and 29 kDa markers are indicated.

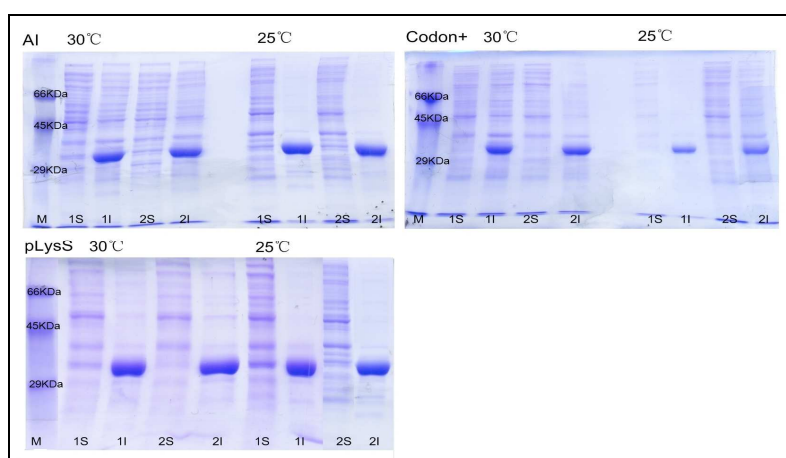


Fig 6. 14: Solubility test for recombinant proteins of pMUT242 in *E. coli* expression strains BL21DE3, BL21 AI, BL21 Codon⁺ and BL21 pLysS.

For each expression strain, two recombinant protein productive transformant colonies were chosen for checking the protein solubility (see methods). Cell pellets from each strain were resuspended and sonicated into two buffers: ① 50 mM potassium phosphate, 1 mM DTT, pH 7.2; ② 50 mM potassium phosphate, 1 mM DTT, pH 7.2, 0.5 mM NaCl. After sonication, the supernatant from centrifugation represents soluble portion while the cell pellets represents the insoluble portion. S: soluble portion. I: insoluble portion. Positions of 66 kDa, 45 kDa and 29 kDa markers are indicated.

Chapter 7 Discussion and Future work

7.1 Final discussion

Proteolytic processing of fungal transcription factors has been reported previously. For example, the *A. nidulans* zinc finger-containing transcription factor PacC undergoes two step proteolytic processing in response to alkaline ambient pH. However, this differs from the case of NmrA where proteolysis negates the activity of a transcription repressor, in that PacC is processed to produce the biologically active form of a transcription activator (Hervas-Aguilar et al., 2007a). Proteolytic processing of a transcription repressor has been reported in Human, and the possibility of regulating transcription factors by target-specific proteolysis has been suggested as a general mechanism (Lin et al., 1995). For example, activation of the nuclear factor κ -B (NF- κ -B) is modulated by its interaction with the repressor protein I κ B- α , and the activity of I κ B- α is itself controlled by ubiquitination and subsequent proteasome-mediated degradation (Brown et al., 1995; Hu et al., 2005; Lin et al., 1995).

Depletion of NmrA under conditions of nitrogen starvation has been reported previously (Wong et al., 2007). In these experiments western blot analysis was used to monitor the levels of NmrA over a four hour period of nitrogen starvation and demonstrated almost complete loss of NmrA. The diminution in the NmrA levels over a time course of nitrogen starvation conditions seen in the western blot experiments was not associated with the formation of proteolytic products corresponding to NM1A, NM1B or NM3. This apparent anomaly can be explained by considering the genotype of the

A. nidulans strain used in the western blot experiment. The strain used contained a recombinant *nmrA* gene situated at the normal locus and under the transcriptional control of the native *nmrA* promoter and the 3'-untranslated region of the *trpC* gene. The recombinant *nmrA* gene encoded an NmrA protein that had a Flag-tag fused to its C-terminus and the NmrA fusion protein was identified in western blots by the use of anti-Flag tag antibodies. Due to the ordered proteolysis of NmrA, the first site to be digested will be in the C-terminal disordered surface loop and this will have the effect of severing the covalent link between the N-terminal NmrA fragments and the Flag epitope. Consequently proteolytic intermediates NM1A, NM1B and NM3 of NmrA would be rendered invisible in a western blot analysis that used an anti-Flag-tag antibody.

The finding that the sequence of the hypothetical protein Q5BAR4 is not highly conserved and only sporadically identified in BLAST searches of closely related species was unexpected. It may be that in this respect *A. nidulans* is atypical and that the ordered proteolysis of NmrA is an unusual aspect of the control of nitrogen metabolite repression confined to this species. However protein BLAST searches reveal that homologues, albeit with substantial sequence divergence, appear to be present in *Gibberella zeae* and *A. terreus*. It is possible that in other species trypsin-like enzymes that are not revealed in BLAST searches with Q5BAR4 fulfil the same function. In this respect it is relevant to note that bovine trypsin has 36.5% identity with Q5BAR4 but was not identified in the top 100 proteins hits in such a BLAST search. Nonetheless bovine trypsin digests NmrA in a manner indistinguishable in SDS-PAGE analysis from PNM1B. *In vivo* confirmation that PNM1B corresponds to Q5BAR4

requires the generation and analysis of an *A.nidulans* strain deleted for the gene AN2366.2. Further *in vitro* proof could be provided by analysing the proteolytic properties of purified recombinant Q5BAR4 encoded by AN2366.2.

It has been speculated previously that NmrA may have an additional role as a redox sensor (Lamb et al., 2003). If the function of a redox sensing ability is to form a link between changes to cellular metabolism and the control of transcription, one might expect that NmrA would be more likely to have its biological effects modulated by binding reduced dinucleotides (Fjeld et al., 2003). The rationale for this argument is that it follows from the high (mM range) NAD(H) concentration and the high NAD^+/NADH ratio (500-700) (Pollak et al., 2007; Williamson et al., 1967) that conversion of NAD^+ to NADH will have the greatest relative effect on the NADH level (Fjeld et al., 2003). In this respect it is important to note that the human protein HSCARG, which belongs to the NmrA-like structural family, binds NADPH with a 360-fold lower K_D than that for NADP^+ (Zheng et al., 2007). Given these observations, a redox sensing role for NmrA is less likely.

Interestingly, the resistance of the N-terminal 32 kDa proteolytic fragments of NmrA (NM1A/B) to further digestion is enhanced *in vitro* by binding oxidised dinucleotides. However, the biological significance of this observation is not clear since the initial proteolytic cleavage event does not appear to be affected by dinucleotide binding and is sufficient to abrogate the ability of NmrA to bind to the AreA Zf. It could be argued therefore, that the dinucleotide binding properties of NmrA have no *in vivo* significance and are simply an evolutionary ‘echo’ of its membership of the superfamily containing the short-chain dehydrogenase-reductases (SDRs) (Lamb et al., 2003). However, the

dinucleotide binding motif GlyXXGlyXXGly characteristic of bi-domain SDRs is replaced by the sequence AsnXXGlyXXAla in NmrA. It appears therefore that, although there have been substantial evolutionary changes to the amino acid sequence; there has been selective pressure for NmrA to retain the ability to bind oxidised dinucleotides. Most of the dinucleotide pool is believed to be protein bound (Pollak et al., 2007) *in vivo*, and this latter observation has led to the caveat that ‘estimates of the actual substrate availability, for example, for NAD(P)⁺-mediated signalling processes are to be viewed with caution, at least, when based upon pyridine nucleotide concentrations in cellular extracts’ (Pollak et al., 2007). As NmrA appears to be produced *in vivo* under most physiological conditions, there is no obvious reason why it should not form part of the cellular pool of protein that binds the majority of the dinucleotide pool *in vivo*. Therefore, it is not unreasonable to infer that a significant proportion of the *in vivo* NmrA pool is likely to have bound NAD⁺ under most physiological conditions. In a highly speculative scenario it is possible that the NM1B+C term protein of NmrA may have an *in vivo* biological role distinct from the repressing role of intact NmrA. If this were the case then in the presence of PNM1A, PNM1B and PNM1C dinucleotide binding may increase the half life of any biological function associated with NM1B+C-term.

This speculative scenario bears some similarity with the site-specific proteolysis of the transcriptional coactivator HCF-1 (Kristie and Sharp, 1990). HCF-1 is cleaved within a proteolytic processing domain (PPD) that contains multiple 20 amino acid reiterations (Wilson et al., 2000). Cleavage within the PPD leads to the production of a family of 100- to 180- kDa N- and C-terminal polypeptides. However the N- and C-terminal

proteolytically-derived polypeptides remain associated. The HCF-1 PPD is proposed to interact directly with other proteins including the FHL2 protein that functions as a transcriptional coactivator/corepressor. The FHL2 protein selectively interacts with non-cleaved HCF-1 and coactivates an HCF-1-dependent promoter (Vogel and Kristie, 2006). Therefore, HCF-1 provides a good example of a mechanism whereby the proteolytic cleavage of a transcriptional coactivator protein can alter its biological activity, even though the proteolytic fragments remain associated.

In conclusion the data lead to the proposition that the dynamic interplay between the production of NmrA and its subsequent ordered proteolysis facilitates a rapid and finely tuned response to changes in the concentration and nature of the nitrogen source supporting growth.

7.2 Future work

One of the most interesting remaining questions is the possible biological function (s) of NM1B+C term of NmrA. A general technique for detecting ligands that interact with NM1B+C term is to construct a specific affinity column by covalently linking NM1B+C-term to a matrix. Potential interacting ligands can be isolated by passing cells free extracts (derived from *A.nidulans* subject to nitrogen starvation) over the affinity matrix followed by elution with a salt gradient. Proteins recovered by this chromatographic process could then be identified by mass spectrometry. It is also of interest to purify PNM1A and PNM1C and to determine their identity by mass spectrometry. Long term goals in this area include the biological confirmation of the mass spectrometry assignments through gene deletion experiments and assay of purified

of recombinant protein. Finally it is also of great interest to determine the temporal induction kinetics for the production of PNM1A, PNM1B and PNM1C particularly as the production of PNM1C appears to be under the control of AreA.

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